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TOXICITY OF COPPER TO DAPHNIDS IN RECONSTITUTED AND NATURAL WATERS

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TOXICITY OF COPPER TO DAPHNIDS IN RECONSTITUTED AND NATURAL WATERS

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ABSTRACT

The toxicity of copper was compared for Daphnia magna cultured in reconstituted versus pond water and fed on trout-pellet versus vitamin-enriched, algal foods. Effects of a chronic copper stress were highly variable when animals were tested in reconstituted waters. This variability is thought to be due to variability in the quality of the distilled-water matrix. The vitamin-enriched algal food was found to be superior to the trout-granule food in culturing D. magna. Control animals lived much longer and test animals were less sensitive to a chronic copper stress. The acute and chronic toxicity of copper was also compared for four species of Daphnia. When tested in pond water and fed vitamin-enriched algae, the two largest species (D. magna and D. pulex) were significantly less sensitive to an acute copper stress than the two smallest species (D. parvula and D. ambigua). There was, however, no significant difference in sensitivity to a chronic copper stress when reduced longevity was used as the index. Application factors for the four species varied from 0.47 to 0.62 and were not significantly different.

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SECTION I

CONCLUSIONS

1. Survival of Daphnia magna was highly variable in Marking's (1969) reconstituted, standard water and medium waters. Most of the variability is thought to be due to variations in the quality of the distilled water. Since longevity was improved by vigorous, overnight aeration of the distilled water, the variable quality of the water was probably due to one or more volatile toxicants.

2. D. magna reared in reconstituted, standard water lost the biramous portions of their swimming antennae about 24 days after birth. Neither the use of an ultrapure standard water, nor vigorous aeration of the distilled water base, prevented the development of this antennal damage. The onset of antennal damage was, however, delayed from day 24 to day 57 by doubling the calcium concentration of the standard-water formula. Antennal damage was never observed in the pond-water system.

3. Unless a methodology can be developed for producing a distilled water of consistently high quality in laboratories whose raw-water supply is of highly variable quality, bioassay research involving the use of reconstituted waters is likely to produce results which are as, or more, variable than results which are produced using natural waters.

4. The vitamin-enriched algal food of Murphy (1970) proved excellent for the maintenance of six species of Daphnia (D. magna, D. ambigua, D. parvula, D. pulex, D. pulicaria, and D. galeata-mendotae). When animals were fed this food, and maintained in pond water, we had no trouble with population depressions or die-offs in our stock cultures over a 2-year period.

5. Survival and reproduction of animals was highly reproducible in both algal, pond-water and trout-granule, pond-water systems under both control and experimental conditions.

6. The two largest species (D. magna and D. pulex) were significantly less sensitive ($P = 0.05$) to an acute copper stress than were the two smallest species (D. parvula and D. ambigua).

7. The 72-hr LC_{50} values for copper were very close, and not significantly different ($P = 0.05$) for D. magna tested on algal- and trout-granule foods in pond water.

8. Interspecies response to an acute chromium stress was much more variable than to an acute copper stress, with D. ambigua being most sensitive and D. pulicaria being least sensitive.

9. The MATC was estimated to be 40 ppb copper for D. magna, D. ambigua, D. parvula, and D. pulex in the pond-water, algal system when longevity was used as the criterion. However, the MATC was estimated to be 10 ppb copper for D. magna tested in the trout-granule, pond-water system.

10. A 3-week time interval is not sufficient to determine the effect of a chronic copper stress on survival of D. magna in pond water. The effect of a chronic copper stress on the three smallest species (D. pulex, D. parvula, and D. ambigua), however, can be evaluated within a 3-week time period.

11. F_1 and F_2 generations of D. magna were no more susceptible, or resistant, to a chronic copper stress than was the parental generation.

12. The MATC, based on longevity, was estimated to be between 5.0 and 7.5 ppb copper for D. magna tested in unaerated, standard water and between 15.0 and 20.0 ppb in aerated, standard water.

13. D. magna exhibited a highly variable response to a chronic copper stress in medium water, with the MATC varying between 10 and 60 ppb. Differences are thought to be due to variations in distilled-water quality.

14. Reproductive response, of four species, to a chronic copper stress was more variable than was longevity in the pond-water, algal system. D. magna did not exhibit a reduction in mean brood size at any concentration in which animals lived long enough to reproduce and did exhibit a stimulation of reproduction up to a concentration of 60 ppb. There was never a stimulatory effect of copper on brood sizes in D. pulex, D. parvula, or D. ambigua. D. pulex exhibited a significant reduction in mean brood size at 80 ppb, D. parvula at 60 ppb, and D. ambigua at 40 ppb ($P = 0.05$)

15. In D. magna, D. pulex and D. parvula, survival was a more sensitive index of a chronic, copper stress than was reproduction. In D. ambigua, survival and reproduction were equally sensitive as indices of a chronic, copper stress.

16. When fed the trout-granule food, D. magna exhibited a reduction in mean brood size at all copper concentrations greater than 10 ppb.

There was, also, a delay in attaining reproductive maturity at higher copper concentration which did not occur in algal-fed animals.

17. The trout-granule food was definitely inferior to the algal food. Animals reared on the trout-granule food exhibited reduced longevity under control conditions and greater sensitivity to copper as measured by either mean brood size or longevity.

18. Daphnia parvula, D. pulex, and D. ambigua all exhibited reductions in the instantaneous rate of population growth ($\bar{r}$) at copper concentrations greater than 40 ppb. Because of the stimulatory effect of copper on brood size in D. magna, $\bar{r}$ did not decline in this species until the chronic copper stress exceeded 60 ppb.

19. Application factors for D. magna, D. pulex, D. parvula and D. ambigua were 0.47, 0.57, 0.62, and 0.59, respectively.

20. Application factors determined for daphnids ($\cong$ 0.5) were much larger than literature values of copper application factors for fish ($\cong$ 0.1).

SECTION II

RECOMMENDATIONS

1. Reconstituted waters should not be recommended for routine bioassay work unless, or until, a methodology can be developed for producing a distilled-water base of uniformly high quality.

2. More work is needed to adequately define the dissolved mineral requirements of daphnids before we can confidently recommend a mineral composition for reconstituted waters.

3. The nutritional adequacy of a food should be demonstrated before it is used in routine bioassay work. It may well be that the same food will not be adequate for different species of cladocerans or even for different species within the genus Daphnia.

4. An application factor of 0.5, based on a 72-hr LC_{50} and an MATC calculated from reductions in longevity, should predict a safe concentration of copper for daphnids which are not exposed to other stresses.

5. A reduction in longevity should be used as the most sensitive index of a chronic copper stress in daphnids.

6. Although D. magna's sensitivity to copper was not significantly different from that of the other species tested, further research is needed to determine whether this is true in relation to other toxicants.

7. Concerted effort should be devoted to determining whether safe concentrations based on chronic bioassay experiments with single species under laboratory conditions are adequate to protect the important components of communities. Laboratory-derived safe concentrations should be tested on more complex systems, such as experimental ponds or streams.

SECTION III

INTRODUCTION

Laboratory studies on the toxicity of chemicals to aquatic organisms may be carried out for a variety of reasons. One of these is to produce information that will contribute to predicting the effects of chemicals on the biota of aquatic ecosystems. This is an extremely difficult objective to achieve because of the inordinately complex nature of ecosystems. Both the abiotic and the biotic components are not only very complex, but they are also highly variable in space and time. If the integrity of an ecosystem is to be protected, concentrations of chemicals must not be allowed to exceed the tolerance of the most sensitive species under those environmental conditions and at those times when it is most sensitive. The difficult task is that of predicting a safe concentration for a large number of species exposed to fluctuating, physical-chemical conditions. Even if one pursues the more limited objective of protecting only certain "important" or "valuable" species, the task is still exceedingly difficult.

Most of the literature dealing with the acute and chronic effects of chemicals on aquatic life is concerned with effects on a few species of fish and on a very few species of invertebrates. Virtually all of the work on planktonic invertebrates (zooplankton) has dealt with a single species, Daphnia magna (Kemp et al., 1971). D. magna has a very limited geographic range in N. America and even within this range it is confined to small bodies of water (Brooks, 1957). Probably, very few aquatic biologists in the U. S. have ever seen a wild specimen of D. magna. Since D. magna is not a frequent component of zooplankton communities, continued use of this species in research designed to formulate water-quality standards can be justified only if it can be demonstrated that D. magna has a sensitivity comparable to that of more widespread, important members of zooplankton communities. One of the objectives of this study was to compare the copper sensitivity of D. magna with that of other, more common, species of Daphnia.

The ability to predict safe concentrations for chemicals is complicated by the necessity to take into account the sensitivity of the species throughout its life cycle. Safe concentrations, ideally, should be based on tests of the sensitivity of a species throughout several generations. This ideal is continuously compromised because of the immediate need for setting water-quality standards and the difficulty in maintaining many species under laboratory conditions. It has been suggested that the ratio between chronic and acute toxicity of a toxicant (the application factor of Mount and Stephan, 1969) is relatively constant for closely-related species or in waters of differing chemical composition.

If this concept were to prove valid, the factor could be used to extrapolate from the acute response of a species to its chronic response. Although there are some published data on application factors in fish, there are no such data available for daphnids. A second objective of the present study was to evaluate the application-factor concept as it applies to daphnids.

Finally, much of the variability in bioassays of heavy metals is known to be a consequence of differences in the chemistry of test waters. It has been proposed (e.g., Marking, 1969; Stephan, 1975) that this variability can be eliminated, or greatly reduced, by using reconstituted waters of known chemical composition in bioassay research. There are no published data, however, to indicate that reconstituted waters are physiologically adequate for the long-term maintenance of any invertebrate animal. The third objective of this grant was to evaluate the adequacy of reconstituted waters as culture media for daphnids.

SECTION IV

METHODS

CULTURE TECHNIQUES

General

Both acute and chronic testing were initiated with new-born daphnids ($\pm$ 12 hrs old). Each animal was maintained, individually, in 40 ml of medium in a 50-ml, Pyrex or Kimax glass beaker. All animals were maintained in environmental chambers at a temperature of 20°C, on a 15-hr photoperiod, at approximately 300 foot candles of illumination.

Each animal was fed and examined daily; in chronic experiments this was continued until all animals were dead (i.e., exhibited no movement). Daily records were maintained for each animal to indicate: molts, age at reproductive maturity, and whether the animal was gravid. Offspring were counted and removed daily. Any abnormalities in appearance were also noted.

Each animal was transferred to fresh medium, in a clean beaker, by eye dropper at 3-day intervals. Tips of eye droppers were cut off to prevent mechanical damage to the larger daphnids. All glassware was cleaned by soaking in 7% nitric acid for at least 24 hrs.

Foods

All stock animals, and animals in most of the experiments, were fed vitamin-enriched algae (*Chlamydomonas reinhardii*). The chemical composition of the medium in which the algae were cultured is given in Table 1. Algae were cultured in Corning 4422 wide-bottomed culture flasks. Each flask contained 1 L of medium and was incubated under the same light-temperature regime as that described for the daphnids. Vitamins were added to the cultures at the concentrations suggested by Murphy (1970). Air, enriched to 10% with CO_2, was bubbled through the cultures, and the cultures shaken, several times each day.

The algae were concentrated from the culture medium by centrifuging through a non-metallic, continuous-flow centrifuge (G. M. Mfg. Corp., El Cajon, CA). The algal concentration was adjusted to a volume such that it gave a reading of 1.5 absorbance units, at a wavelength of 665 millimicrons, in a spectrophotometer. Three drops of this concentrate were fed to each animal daily. This averaged 0.34 mg, dry weight, or 0.31 mg, ash-free dry weight, of algae per feeding.

TABLE 1. COMPOSITION (MG/LITER) OF THE MEDIUM USED IN CULTURING *Chlamydomonas reinhardii*

Constituent	Concentration (mg/l)
$NaNO_3$	5.0
$CaCl_2.2H_2O$	0.25
$MgSO_4.7H_2O$	0.75
K_2HPO_4	0.75
KH_2PO_4	1.75
NaCl	0.25
EDTA	0.05
$FeSO_4$	0.005
H_3BO_3	0.012
$ZnSO_4.7H_2O$	0.009
$MnCl_2.4H_2O$	0.001
MoO_3	0.0007
$CuSo_4.5H_2O$	0.0016
$Co(NO_3)_2.6H_2O$	0.0005
Calcium pantothenate	7.0
B_{12}	0.0008
Thiamin	0.6
Riboflavin	0.4
Nicotinamide	1.3
Folic acid	3.3
Biotin	0.3
Choline	5.0
Putrescine	0.3

One series of chronic tests was carried out using the trout-granule, dried-grass, food developed at the Duluth National Water Quality Laboratory (Biesinger and Christensen, 1972). Glencoe trout-starter granules and the dried grass, which was actually a commercially-available health food called Cerophyl (Cerophyl Lab. Inc., Kansas City, MO), were kindly provided by K. B. Biesinger. The trout granules were ground in a mortar and pestle and sieved, dry, through #20 plankton netting to remove the larger particles. The food suspension contained 0.4 g of trout granule and 0.02 g of Cerophyll per liter of pond water, was made up fresh weekly and kept in a refrigerator. Each animal was fed 114 microliters of the mixture daily. This averaged 0.42 mg, dry weight, and 0.31 mg, ash-free dry weight, of food per feeding. By coincidence, the ash-free dry weights of algae and trout granule foods, per feeding, were identical.

Culture Media

Daphnids were reared in three different kinds of water at various times during the study. Most of the work, both acute and chronic, was done in a local pond water. Considerable time and effort, however, was expended in trying to develop a "reconstituted" water that would be satisfactory for the rearing of daphnids. These reconstituted waters were created by dissolving reagent-grade salts in double-, glass-distilled water. The salt concentrations are those originally suggested by Marking (1969) for waters that he designated as "standard" and "medium" water. Chemical characteristics of the pond and the two reconstituted waters are presented in Table 2. Reconstituted waters were also made up from double-distilled water that had been further processed by filtration. Both a Barnstead, D-8904, Organic Removal Filter and an Aqua-Pure, P50-1, Taste-and-Odor-Removal Filter (Cuno Eng. Corp., Meriden, Conn.) were tested to see whether or not they would improve the quality of the reconstituted waters. Finally, an ultrapure water, kindly provided by Dr. William Hoyle of the Miami University Chemistry Department, was tested in an attempt to improve the quality of the reconstituted water. This ultrapure

TABLE 2. GENERAL CHEMICAL CHARACTERISTICS OF THE THREE TEST MEDIA

Water	pH range	Total Hardness	Total Alkalinity
Standard	6.8-7.9	40-48	30-35
Medium-Hard	8.2-9.5	160-180	110-120
Local Pond	8.2-9.5	130-160	100-118

water was produced by processing water from a still through, in sequence, a mono-bed deionizer, an alkaline permanganate distillation and two glass stills.

TOXICANT INFORMATION

Copper was added to the culture medium as reagent grade $CuSO_4 \cdot 5H_2O$. A fresh stock solution of 100 ppm Cu was made up in double, glass-distilled water on each day of use. Copper content of the double-distilled water was less than 1.0 ppb and was determined with a Model 453 Instrumentation Laboratory atomic-absoprtion spectrophotometer using an APCD-MIBK extraction (Analytical Qual. Control Lab., 1971) and the method of standard additions (Christian and Feldman, 1970). Test concentrations were prepared by transferring appropriate aliquots of the 100 ppm stock solution to the test media with a microliter pipette. Concentrations of copper in the test media were monitored by atomic-absorption spectroscopy.

TECHNIQUES FOR MONITORING THE COPPER STRESS

Effect of Copper on Longevity

Differences in longevity were determined by following each cohort until all individuals had died. Differences in survival curves were analyzed by chi-square analysis (Mantel, 1966) in a computer program developed by, and available from, Carl Hacker, Department of Public Health, University of Texas, Austin, Texas.

Effect of Copper on Reproduction

In daphnids, not exposed to a toxic stress, reproductive performance is largely determined by temperature and food resources. Temperature determines age at sexual maturity, longevity, and frequency of reproduction; food resources determine brood sizes and vitality of the young. Temperature and food resources were the same for all animals in the present study, therefore differences in reproductive performance can be attributed to genetic differences or to the effects of exposure to differing copper concentrations.

Reproductive output can be affected directly by a toxicant (causing a change in brood size or frequency) or, indirectly, by shortening the life-span of the female (fewer broods) or changing the age at reproductive maturity. Studies which do not entail frequent examination of individual animals do not provide information which will allow the

investigator to discriminate between direct and indirect effects of the toxicant on reproduction.

The impact of copper on brood sizes was evaluated by a randomized, complete-block design, analysis of variance and Duncan's new multiple range test (Duncan, 1955). These analyses were run, consecutively, from the second brood, through each brood, until the last brood was produced in the experiment. The technique permits an evaluation of the impact of copper on cumulative mean brood size at any point in time throughout the entire chronic bioassay.

Effects of Copper on the Instantaneous Rate of Population Growth

Another way to evaluate the impact of a stress on a species is to calculate its effect on the rate at which the species can increase in numbers. This can be estimated by calculating $\underline{r}$, the instantaneous rate of population growth, which is equal to the birth rate minus the death rate. If one plots the population density, on a logarithmic scale, against time, on an arithmetic scale, the slope of the line is $\underline{r}$. This instantaneous rate is affected by both reproductive performance and longevity. I have calculated $\underline{r}$ for each cohort in each chronic study, using a life-table computer program (C. Hacker, Dept. of Public Health, University of Texas, Austin, TX) by solving for r in the equation:

$$1 = \Sigma P_i \cdot m_i \cdot e^{-ri}$$

where e is the base of the natural logarithm, $\underline{r}$ is the instantaneous rate of population growth, P_i is the proportion of female survivors of age i, m_i is the mean number of progeny per female of age i, and i is in days. Significant differences between $\underline{r}$-values were determined by randomized, complete-block design, analysis of variance and Duncan's new multiple range test.

SECTION V

RESULTS AND DISCUSSION

CHEMICAL CHARACTERISTICS OF TEST WATERS

Reconstituted Waters

Reconstituted-water cultures were monitored over 3-day intervals to document extremes in pH and dissolved oxygen. In standard water, pH varied between 6.8 and 7.9. Oxygen concentrations varied between 7.5 and 8.3 ppm (83 to 90% saturated). In medium water, pH varied between 8.2 and 9.5 and oxygen between 7.8 and 13.5 ppm (85 to 145% saturated). As would be expected, due to the interaction between photosynthesis and respiration, highest pH and O_2 values occurred at the end of the light period and lowest values at the end of the dark period. The standard water had a total hardness of 40-48 and a total alkalinity of 30-35 ppm, as $CaCO_3$. The medium water had four times the salt concentrations of standard water (Table 2) and, therefore, the hardness and alkalinity was four times greater.

Pond Water

Pond water was similar in alkalinity and hardness to the reconstituted medium water, having a total alkalinity ranging between 110-120 ppm and a total hardness ranging between 130-160 ppm, as $CaCO_3$. Oxygen concentrations varied between 8.7 and 11.4 ppm (95-125% saturated) and pH varied between 8.2 and 9.5 in the pond-water, algal system. Variation was less in the trout-pellet, pond-water system. Oxygen extremes were 7.3 to 7.9 (90-98% saturated) and pH varied from 7.9 to 8.0. The differences between the two systems are due to the presence or absence of significant numbers of algae.

TOXIC CONCENTRATIONS IN TEST VESSELS

The concentration of a toxicant gradually decreases in static test systems. The 72-hr renewal of culture medium in the present study was designed to minimize this reduction in toxicant concentration. Table 3 presents data on measured concentrations of total copper in test beakers after 72 hrs of incubation under test conditions (i.e., each beaker contained a test animal which was fed daily). Each value is for a single beaker when nominal concentrations were above 10 ppb. The 10-ppb concentrations were extracted into MIBK and each value in the table is for a

TABLE 3. COPPER CONCENTRATIONS IN TEST BEAKERS AFTER 72 HOURS OF INCUBATION

Nominal Copper Concentration (ppb)	Measured Copper Concentrations (ppb)						Mean (ppb)	% Loss
10	5.9	3.6	8.3	7.6	8.4		6.8	32
20	15.8	15.4					15.6	22
30	27.0	27.0	33.7	29.3	31.0	29.0		
	19.3	16.4	17.6	18.1	22.8	22.1	24.4	19
50	49.7	44.7	43.8	47.6	50.2	52.9		
	46.0	47.6	49.0	45.3	52.7	51.0	48.4	3
60	47.8	50.2	51.1	55.2	51.7	53.3	51.6	24
100	76.8	88.0	79.5	87.0			82.8	17

100-ml aliquot taken from a composite of three test beakers. Copper loss over the 72 hrs ranged from a mean of 32% for the 10 ppb concentration to a mean of only 3.2% for the 50-ppb concentration. Losses were higher from concentrations both above, and below, 50 ppb.

RECONSTITUTED WATERS AS CULTURE MEDIA FOR DAPHNIDS

Standard Water

Our initial experiments with standard water were very encouraging. Figure 1 illustrates survival curves for D. magna reared in standard water that had been reconstituted from double-distilled water processed through a Barnstead Organic-Removal Filter. Animals were fed the algal diet and each curve is for a cohort of ten, newborn animals. The first three experiments (6/8, 7/4, and 8/12) produced very similar results, with mean longevities of 30.5, 30.9, and 30.2 days. Subsequent to these experiments, however, we have had highly variable results in attempting to maintain D. magna in standard water. Figure 1 depicts this variability in survival.

The original standard water was made from double-distilled water that had been processed through a "used" Barnstead filter. When the problem of reduced longevity developed, it seemed probable that the cartridge had become saturated and was leaching some toxic substance back into the water. Upon ordering new cartridges, we discovered that they were saturated with formalin to inhibit the growth of microorganisms. Barnstead then provided us with cartridges to which no formalin had been added. Water filtered through these cartridges was even more toxic to daphnids (Fig. 1), mean longevity dropped to 9.5 days. Obviously such cartridges would have to be conditioned by passing water through them for some unknown time period if they were to be used for our purposes. Water processed through an Aqua-Pure filter was more compatible with daphnids, but such water caused a precipitation of copper from our 100 ppm stock solutions. Furthermore, water passed through an Aqua-Pure filter exhibited a five-fold increase in the concentration of phthallate esters according to an analysis performed for us by the Organic Analysis Section, Methods Development and Quality Assurance Research Laboratory, E. P. A., Cincinnati, Ohio. Therefore, we did not attempt any further research with this filter. We also tried a Barnstead, standard deionizing filter; daphnids died within a few hours in standard water made up from water filtered through such a cartridge. It was becoming obvious that cartridges, or filters, not only remove substances from water but that they are also likely to add substances to the water and that some of these are toxic to daphnids. This defeats one of the main objectives of using reconstituted water, i.e., having a reproducible, chemically-defined medium.

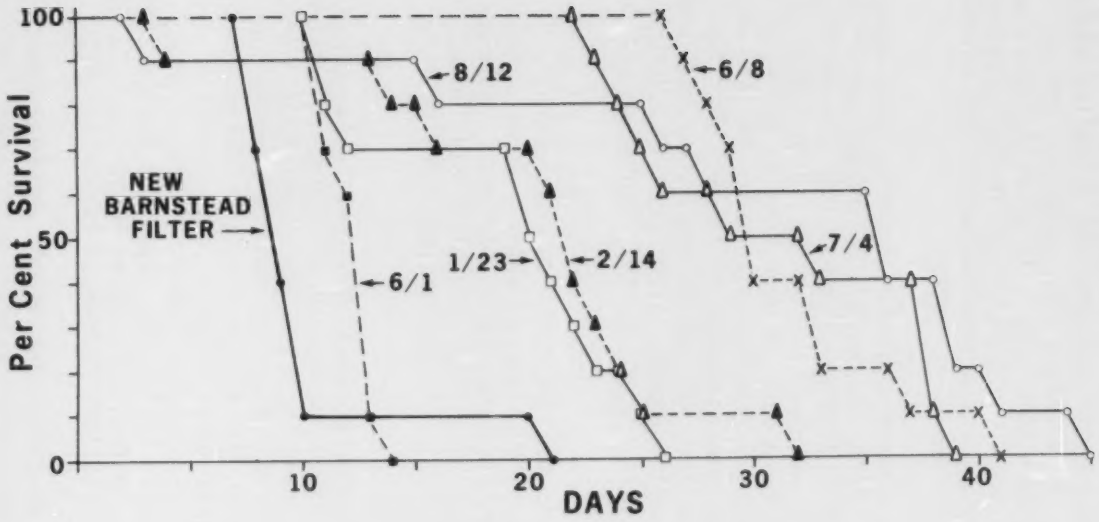

Figure 1. Survival of Daphnia magna fed algae in unaerated filtered, standard water

It seemed probable that any toxic substance, which was passing through two distillations, was volatile. Therefore, we vigorously aerated our unfiltered, double-distilled water overnight before adding the salts. Daphnids did live longer in this aerated, standard water. This is evident in Fig. 2, which shows survival curves for pairs of cohorts of D. magna which were cultured simultaneously in standard water made up from aerated, and unaerated, distilled water. The difference is not due to O_2 or pH; the salts are stirred into the distilled water with a magnetic stirrer and this is sufficient to create O_2 and pH values comparable to the overnight aeration. The initial O_2 for both waters was at saturation and the pH was 7.4. Apparently, however, the vigorous, overnight aeration does reduce the concentration of some chemical or chemicals which decrease the life span of D. magna.

A new problem, however, developed during this phase of the reconstituted water study. Daphnids reared in standard water, made from unfiltered, aerated, double-distilled water, lost the biramous portions of their swimming antennae about 24 days after birth. Loss of the antennae was preceded by the appearance of a dark spot at the base of the biramous portion of the antennae. During the next molt, the antennae broke and were missing throughout the remainder of the animal's life. This did not occur in any of the earlier experiments when water had been filtered through Barnstead, organic-removal cartridges. Possibly, the cartridges removed some toxicant from the water, or, leached something beneficial into the water.

In an attempt to eliminate the antennal damage, "ultra-pure" water was used as a base for standard water. Animals maintained in standard water made up from ultra-pure water did not live longer than animals in standard water made from aerated, double-distilled water (Fig. 3). Furthermore, they also lost the terminal portions of their antennae about 25 days after birth.

Murphy (1970) reported that he successfully reared daphnids in water that had only calcium acetate in solution. The animals obtained all other mineral requirements from their algal food. Since Murphy's culture water contains about twice as much calcium as does Marking's standard water, I doubled the calcium sulfate content of standard water to determine whether that might prevent the antennal damage. Doubling the calcium concentration does not prevent antennal damage, but it does delay it from about day 24 to day 57. Doubling the calcium content of standard water may also markedly increase longevity. In fact either, aeration or increasing calcium, increases longevity (Figs. 2 and 3).

Murphy (1970) does not mention any antennal damage in his cultures. He says (personal communication) that, under the "best" conditions,

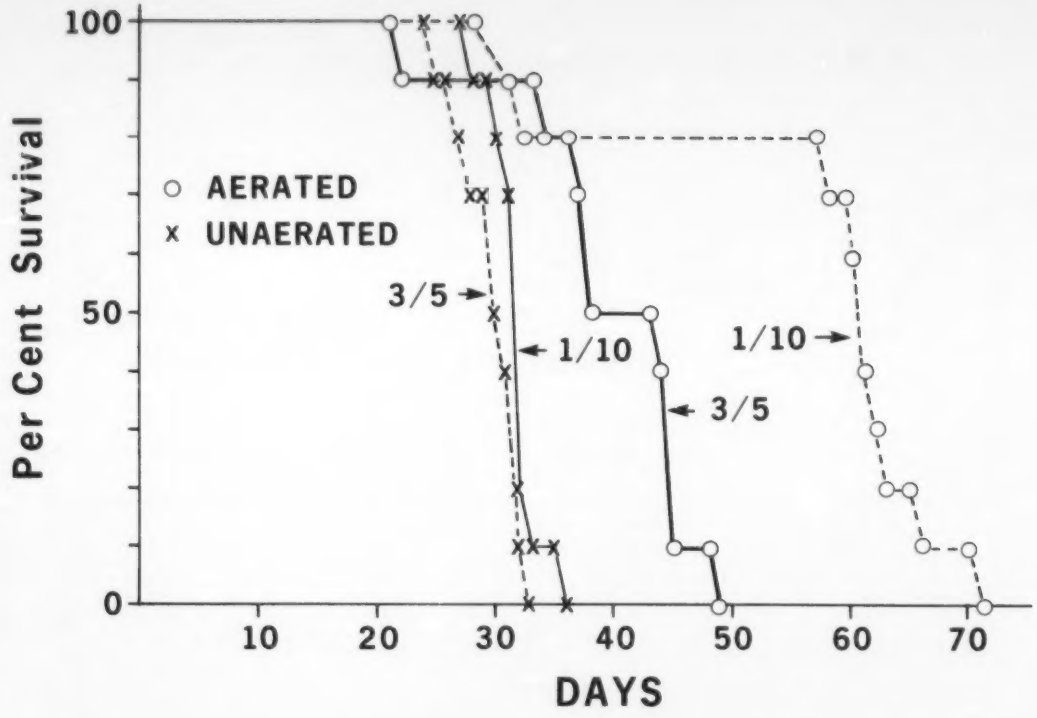

Figure 2. Survival of Daphnia magna in unfiltered aerated versus unaerated standard water

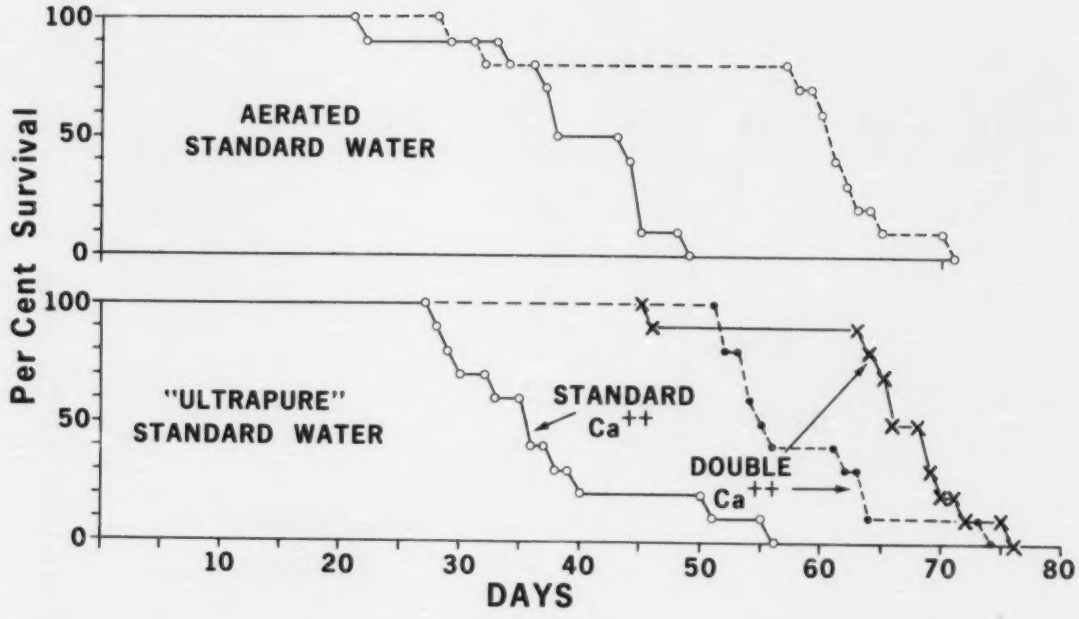

Figure 3. Survival of Daphnia magna in unfiltered versus aerated ultrapure standard water

D. magna has a life span of about two months at 20°C. It may be, then, that his animals die before the antennal deformity can develop.

Medium Water

Efforts to culture D. magna in medium water followed the same trend as described for standard water. Figure 4 illustrates the progressive reduction in longevity that we observed in our medium-water cultures. Animals reared in filtered, unaerated medium water live considerably longer than animals reared in filtered, unaerated standard water and, under the best condition, may live as long as animals in pond water. Figure 5 depicts survival curves for 3 cohorts of ten animals initiated on the same day in filtered, unaerated standard, medium, and pond water. All were fed vitamin-enriched algae. It should be pointed out that the survival curves for the reconstituted waters depicted in Fig. 5 are the best that were observed and that the pond-water survivorship curve was the poorest of all observed during this study. The reason for greater longevity in medium water, compared to standard water, is unknown. There are, at least, three possible causes: (1) the low salt concentration, in some way, directly reduces longevity, (2) additional trace elements are added, as contaminants, with the reagent-grade salts in the medium water, or (3) the higher salt concentrations in the medium water may complex some toxic substance or substances present in the distilled water. Possibility number 2 seems improbable, since the animals are fed algae which have been cultured in a medium containing trace elements (Table 1). If possibility number 1 is true, one would expect a more rapid turnover of daphnid populations in soft-water lakes than in hard-water lakes. To the best of my knowledge, this possibility has never been suggested, or tested. The fact that aeration increases longevity in standard water suggests that possibility number 3 may be the explanation. Allen Maki, Environmental Water Quality Research Department, Proctor and Gamble, Cincinnati, Ohio, states (personal communication) that they have discontinued the use of standard water in their bioassay work because of poor survival and reproduction of the daphnids in standard water.

Summary

We have found it difficult to maintain viable stock cultures of Daphnia in reconstituted waters. We have had to replenish our stock animals in reconstituted water, from time to time, with animals from our pond-water stocks. At no time, over a 2-year period, did we have any difficulty in maintaining daphnids in our pond-water, algal system. I suspect that life-history statistics for daphnids reared in reconstituted waters made up in different laboratories, or at different times in the same laboratory, may be as, or more, variable than in different, natural waters. I believe that an adequate, reproducible, reconstituted

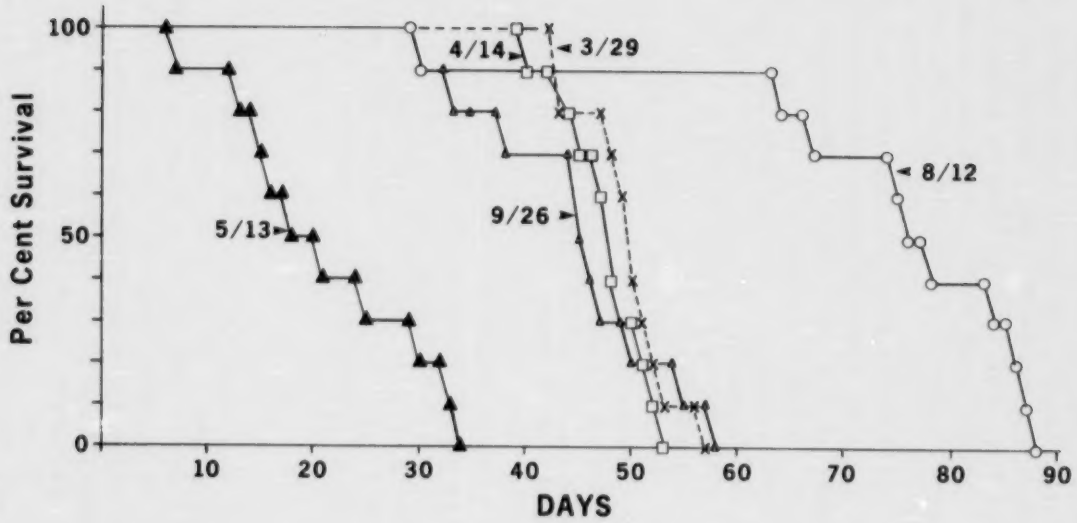

Figure 4. Survival of Daphnia magna fed algae in filtered, unaerated medium water

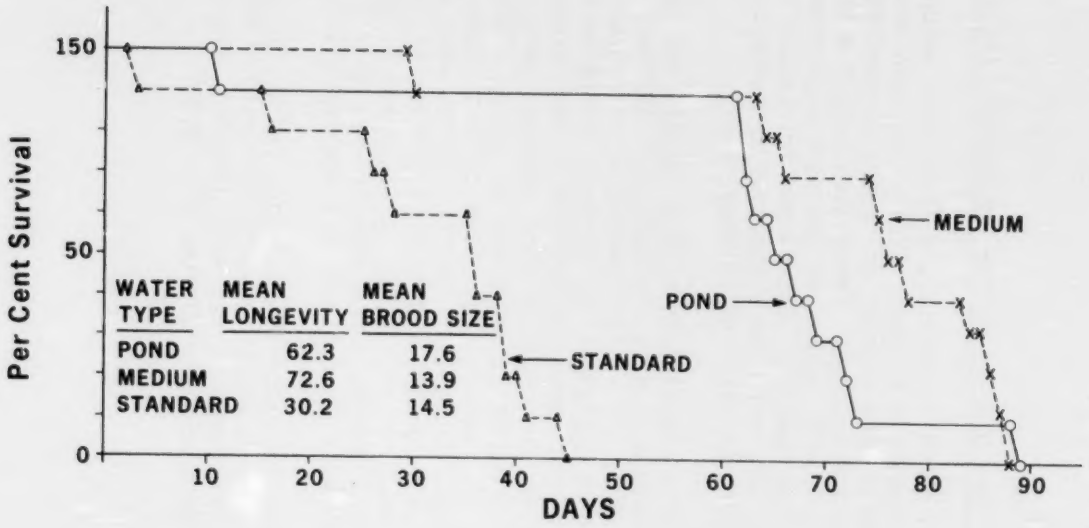

Figure 5. Comparative survival and reproduction of Daphnia magna in filtered, unaerated standard, medium, and pond water

water can be developed if a consistently high-quality distilled water can be produced. I suspect, however, that, given the quality of distilled water available to most laboratories, reconstituted waters are likely to create more problems in acute chronic toxicity studies than they solve. In any event, much more effort must be spent in the development and testing of reconstituted waters before they can be recommended for routine bioassays.

ACUTE TOXICITY OF COPPER

Relative Sensitivity of Four Species in the Pond-Water, Algal System

On day 3 of each chronic bioassay, a 72-hr LC_{50} value was calculated by probit potency analysis (Finney, 1971). In each experiment, cohorts of 10 newborn animals were started at copper concentrations of 0, 20, 40, 60, 80, 100, 120 and 140 ppb. Three such experiments, started at different times, were run for D. magna and two such experiments were run for each of the other three species. Inter- and intraspecific comparisons of LC_{50} values were made only when the respective concentration-response curves were demonstrated to be parallel (Finney, 1971).

Data from the three experiments with D. magna, and the two tests for D. ambigua, were combined to give a single best estimate of an LC_{50} value for each species since all of the concentration-response curves were parallel. In the case of D. pulex and D. parvula, concentration-response curves for the two experiments were not parallel. One concentration-response curve for each of these species was, however, parallel to the concentration-response curves of D. magna and D. pulex. Potency analysis was used to compare the LC_{50} values from those concentration-response curves which were parallel (Table 4). The LC_{50} values were not significantly different ($P = 0.05$) for the two largest species (D. magna and D. pulex) nor for the two smallest species (D. parvula and D. ambigua). The two largest species were, however, significantly less sensitive to an acute copper stress than were the two smallest species (Table 4).

The acute toxicity of copper to D. magna and D. ambigua was also tested in filtered, unaerated, standard water (Table 5). Variability was quite low, concentration-response curves were parallel, and the mean 72-hr LC_{50} values were not significantly different ($P = 0.05$).

TABLE 4. ACUTE TOXICITY (72-HR LC_{50} IN PPB) OF COPPER TO FOUR SPECIES OF *Daphnia* IN THE POND-WATER, ALGAL SYSTEM

D. magna	*D. pulex*	*D. parvula*	*D. ambigua*
86.5[a]	86.0	72.0	67.7[b]
	54.0[c]	57.0[c]	

LC_{50} values sharing an underline are not significantly different.

[a]Calculated from the pooled data of 3 experiments.

[b]Calculated from the pooled data of 2 experiments.

[c]LC_{50} values from non-parallel dose-response curves which were not used in the analyses for significant differences.

TABLE 5. ACUTE TOXICITY OF COPPER AND CHROMIUM TO D. magna AND D. ambigua IN UNAERATED FILTERED, STANDARD WATER

Species	n	Metal	Mean 72-Hr LC_{50} (ppm)	Standard Deviation
D. magna	4	Cu	13.5	2.72
D. ambigua	3	Cu	12.4	1.46
D. magna	5	Cr	5.2	2.80
D. ambigua	6	Cr	1.7	1.20

Toxicity to D. magna in the Three Test Waters

Table 6 illustrates the effect of changes in water chemistry on the acute toxicity of copper to D. magna. The expected, inverse relationship between toxicity and alkalinity is apparent. It is also obvious that subtle, unidentified changes in water chemistry can markedly affect toxicity. The 72-hr LC_{50} values of animals tested in filtered, unaerated-unfiltered and aerated-unfiltered standard water were 13.5, 25.2 and 31.6 copper, respectively. Acute toxicity of copper in medium water was more variable than in pond water, but the means were not significantly different (P = 0 05).

TABLE 6. ACUTE TOXICITY OF COPPER TO ALGAL-FED D. magna IN DIFFERENT WATERS

Water	n	Mean 72-Hr LC_{50} (ppm)	Range (ppm)	Standard Deviation
Unaerated, Filtered Standard	4	13.5	10.1-16.5	2.72
Unaerated, Unfiltered Standard	2	25.2	24.9-25.5	0.42
Aerated, Unfiltered Standard	1	31.6	-	-
Medium	7	78.2	56.8-101.4	16.60
Pond	3	85.1	81.6-88.8	3.63

Effect of Food Type on Sensitivity of D. magna

Contrary to expectation, the two foods tested did not affect the acute toxicity of copper to D. magna in pond water. The mean 72-hr LC_{50} for animals fed algae daily was 85.1 ppb (n = 3; SD = 3.6) and for animals fed the trout-granule diet it was 83.4 ppb (n = 3; SD = 2.8). These were not significantly different (P = 0.05).

Acute Toxicity of Chromium

Sensitivity to an acute chromium stress was much more variable than to an acute copper stress (Table 7). However, all of the concentration-response curves were parallel, both intra- and interspecifically. D. ambigua was much more sensitive to chromium in pond water than were the other three species. It was also more sensitive than D. magna in filtered, standard water (Table 6). Apparently, the similarity of response by various species of Daphnia to copper stress does not necessarily hold for other stresses.

TABLE 7. ACUTE TOXICITY OF CHROMIUM TO FOUR SPECIES OF Daphnia IN THE POND-WATER, ALGAL SYSTEM

Species	n	$\bar{x}$ 72-hr LC_{50} (ppm)	Standard Deviation
D. magna	2	42.1	8.49
D. ambigua	2	7.7	1.34
D. galeata	2	65.6	1.75
D. pulicaria	2	110.8	30.10

CHRONIC TOXICITY OF COPPER

Effect of a Chronic Copper Stress on Longevity

Comparative Sensitivity of the Four Species--

Survival curves for each chronic test on each species are presented in Figs. 6 through 9. All of these tests were run in the pond-water, algal system. The results of chi-square analyses of these survivorship curves are encouragingly consistent. Survivorship curves at 20 and 40 ppb were never significantly different (P = 0.05) from those of control animals. With one exception, all survival curves at concentrations of 60 ppb, or above, were significantly different from those of control animals. The

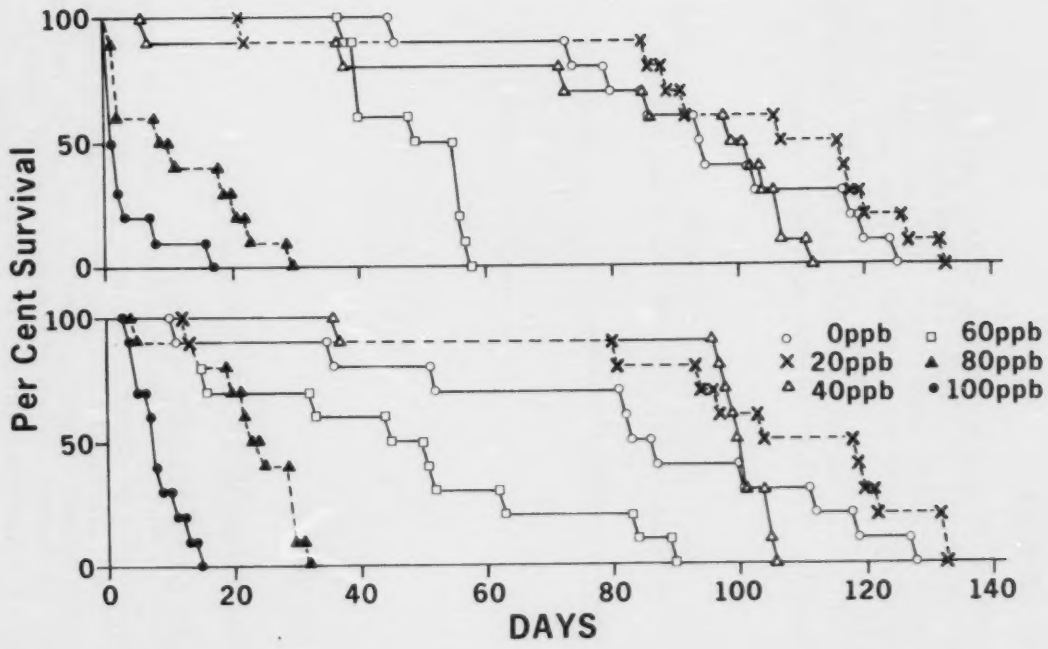

Figure 6. Survival of Daphnia magna exposed to chronic copper stresses in the pond-water, algal system (Experiments 1 and 2)

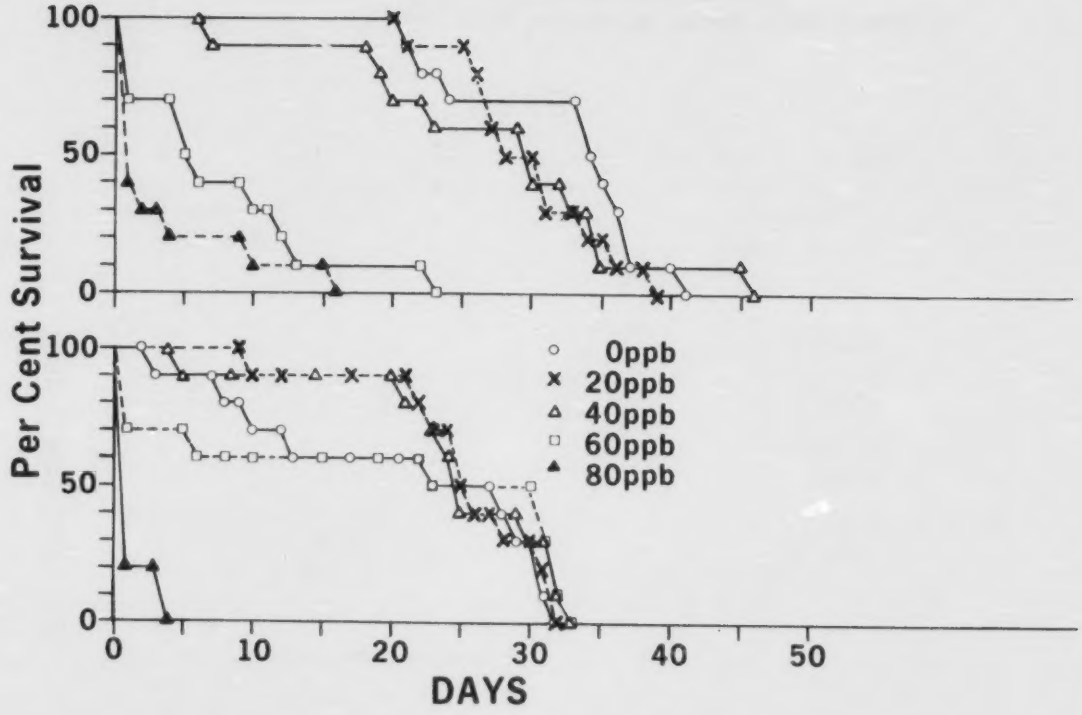

Figure 7. Survival of Daphnia ambigua exposed to chronic copper stresses in the pond-water, algal system (Experiments 1 and 2)

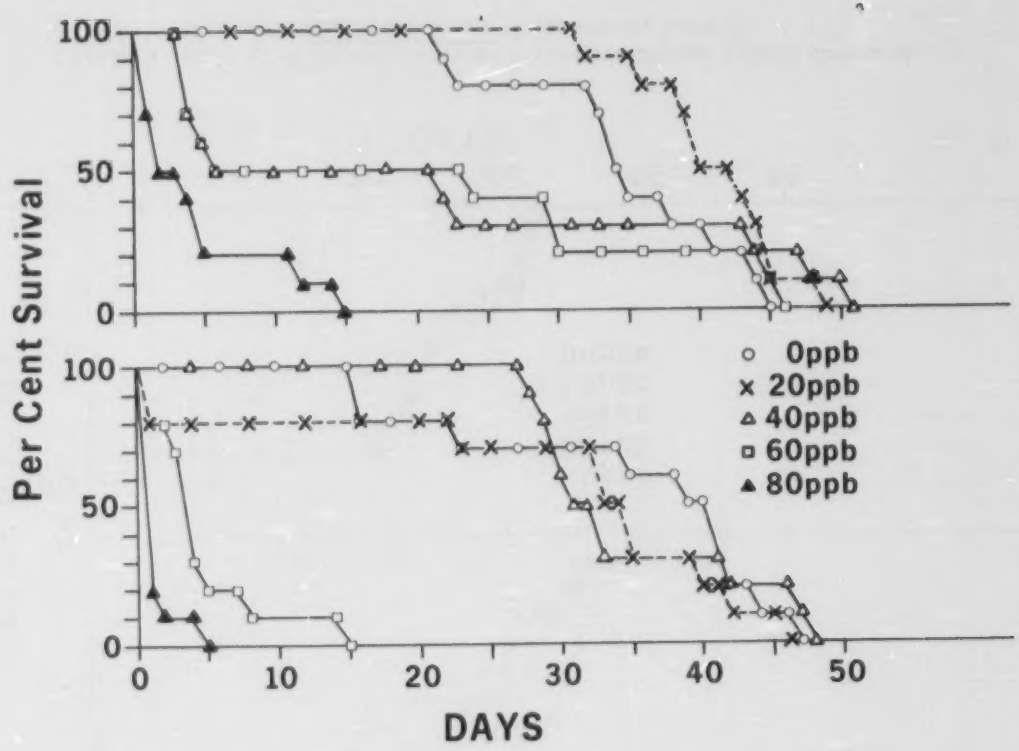

Figure 8. Survival of Daphnia pulex exposed to chronic copper stresses in the pond-water, algal system (Experiments 1 and 2)

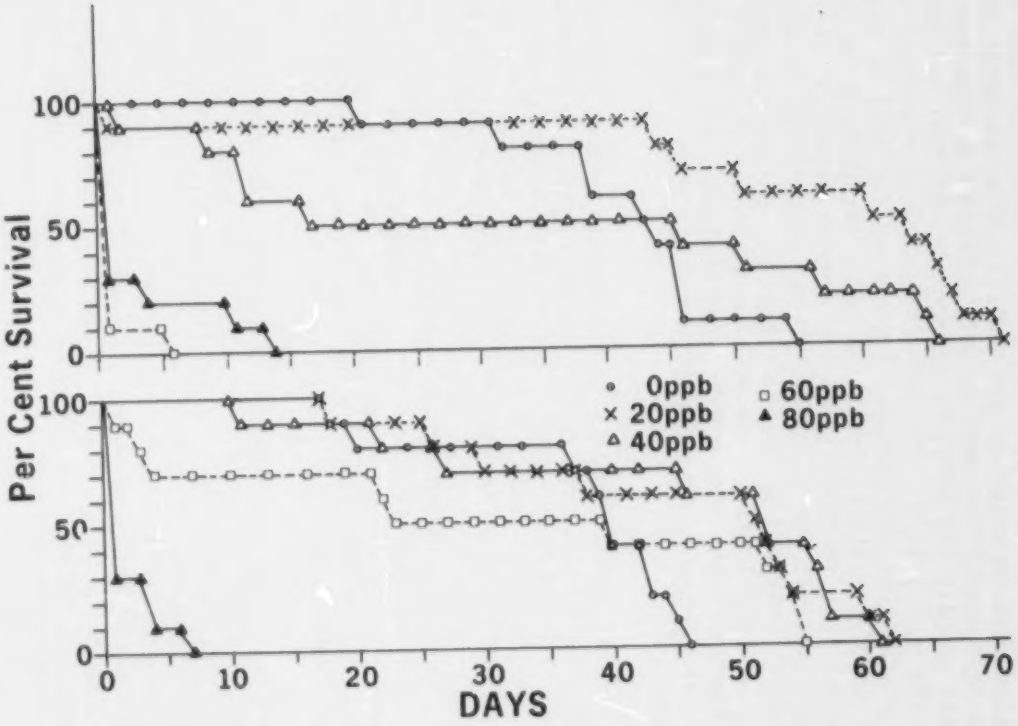

Figure 9. Survival of Daphnia parvula exposed to chronic copper stresses in the pond-water, algal system (Experiments 1 and 2)

one exception is the second chronic test with D. ambigua where survival differed from that of control animals only at 80 and 100 ppb. It should be pointed out that the longevity of all animals, including the controls, was considerably reduced in experiment number 2 with D. ambigua (Fig. 7). This disparity between experiments did not occur with the other 3 species.

The reproducibility of life-history statistics is especially evident in the three chronic toxicity experiments with D. magna. This is evident from the data in Table 8. The third chronic experiment was performed at a later date to check on the toxicity of a new batch of copper sulfate. This reproducibility implies both a rather uniform gene pool in the experimental animals (relative to copper sensitivity) and good reproducibility in the test system. This being the case, I feel that it is reasonable to assume that the somewhat greater variability in the response of the other 3 species reflects greater genetic variability in the test populations rather than variability in the physical-chemical characteristics of the test system. The probability that most of the variability is in the organisms is enhanced by the fact that D. ambigua, D. parvula, and D. pulex were collected locally from wild populations, whereas D. magna was obtained from a culture that had been maintained under laboratory conditions for many years and was probably started from a very few parthenogenetic females.

If most of the variability is in individual-animal response, rather than in test conditions, one gets a better impression of population response by combining the survivorship curves from the separate experiments for each species into combined curves. This is done for each of the four species in Figs. 10 through 13. In these curves, the more rapid die-off of animals in the 60 ppb copper solution is clearly evident. It is obvious that there is a significant increase in toxicity between 40 and 60 ppb. If current hypothesis is correct (Stiff, 1971; Pagenkopf et al., 1974), there must have been a saturation of the complexing capacity of the pond water within this range and a significant increase in the concentration of cupric ions.

It is evident, from these experiments, that the impact of a chronic copper stress on survival is not significantly different for these 4 species of Daphnia. If, however, the chronic experiments had been terminated at the end of 3 weeks, as done by Biesinger and Christensen (1972), D. magna would have appeared to be less sensitive since the die-off at 60 ppb did not begin until the experiment had progressed beyond day 30 (Fig. 10). Obviously, daphnids very rarely, or never, attain their physiological life expectancy in nature and one could speculate that it is really not worth the effort to carry chronic experiments through to the death of the last animal. It is clear, however, that D. magna was under stress in these experiments at a copper concentration of 60 ppb, and that this

TABLE 8. VITAL STATISTICS FOR THREE CHRONIC COPPER TOXICITY TESTS WITH *D. magna* IN THE POND-WATER, ALGAL SYSTEM

Copper Conc (ppb)	$\bar{x}$ Longevity (days)			Total Young			$\bar{x}$ Brood Size		
	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3
0	79.1	93.2	96.7	2314	3223	3287	10.6	13.1	12.3
20	99.6	99.7	91.7	3180	3863	2850	12.5	14.6	12.9
40	94.2	82.7	106.0	3329	3394	3110	13.3	15.2	13.1
60	45.3	48.8	69.0	1689	2150	2328	13.7	16.5	13.2
80	22.2	11.0	20.6	464	364	696	10.8	14.0	14.2
100	7.5	2.7	3.0	0	26	14	-	13.0	7.0

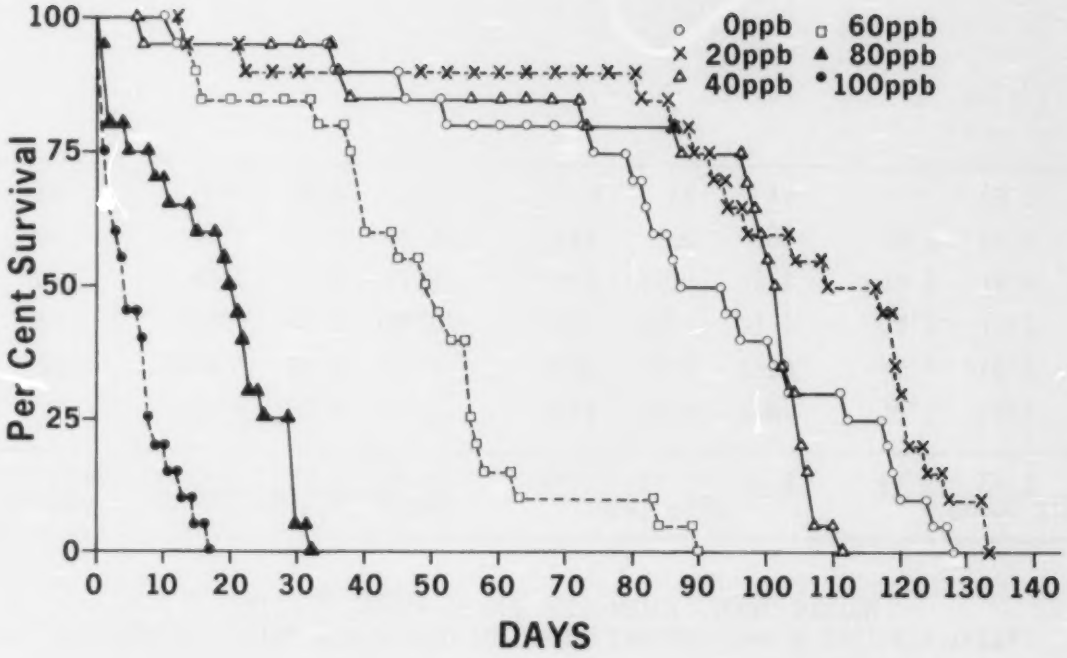

Figure 10. Combined survivorship curves (Experiments 1 and 2) for Daphnia magna exposed to chronic copper stresses in the pond-water, algal system

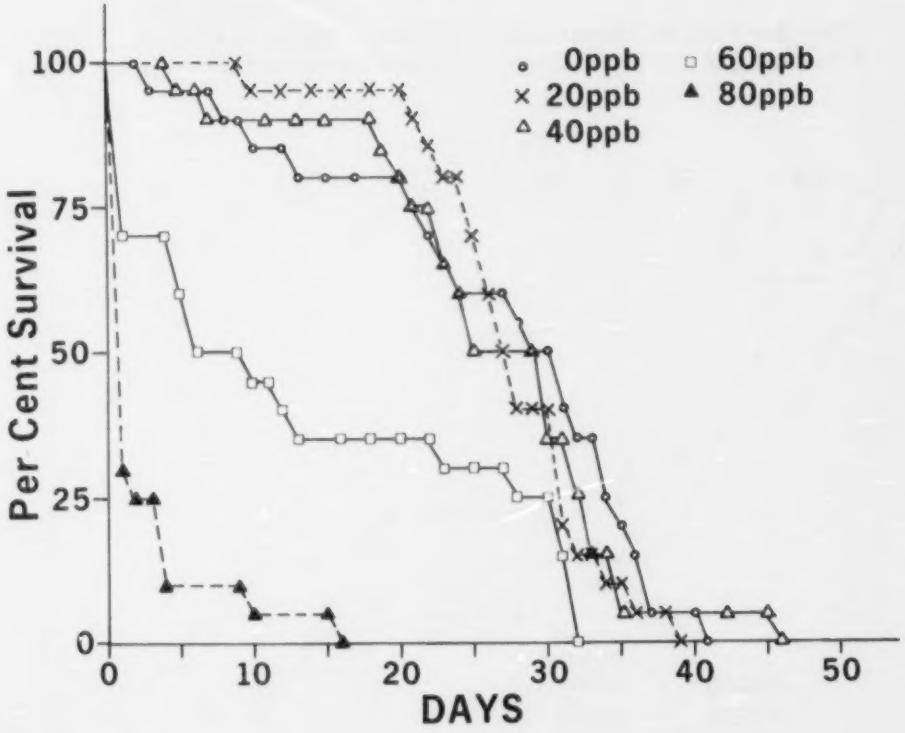

Figure 11. Combined survivorship curves (Experiments 1 and 2) for Daphnia ambigua exposed to chronic copper stresses in the pond-water, algal system

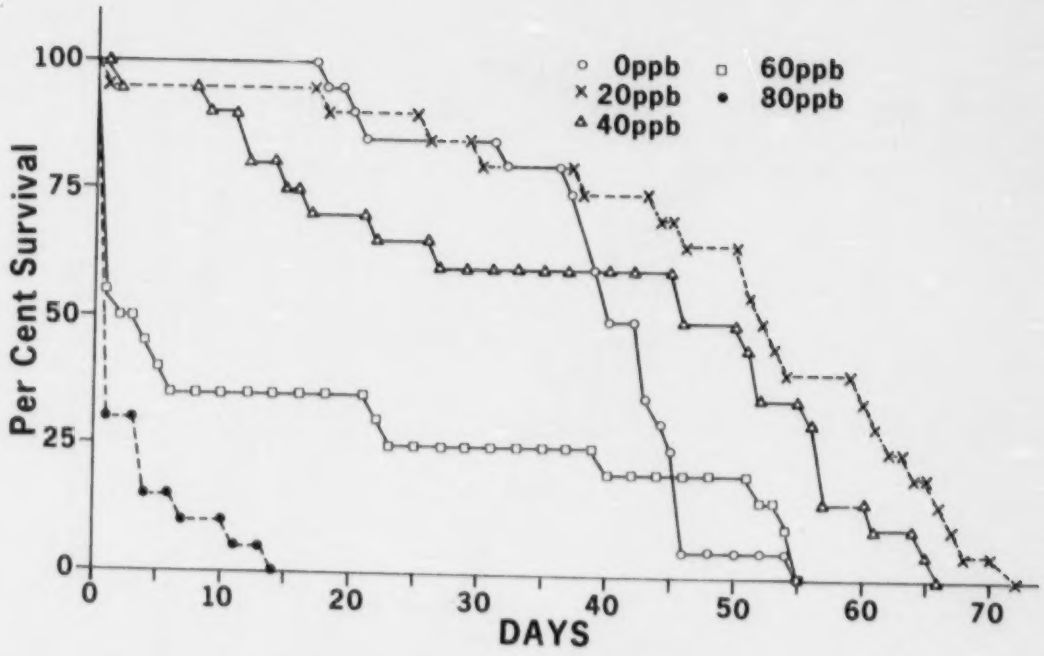

Figure 12. Combined survivorship curves (Experiments 1 and 2) for Daphnia parvula exposed to chronic copper stresses in the pond-water, algal system

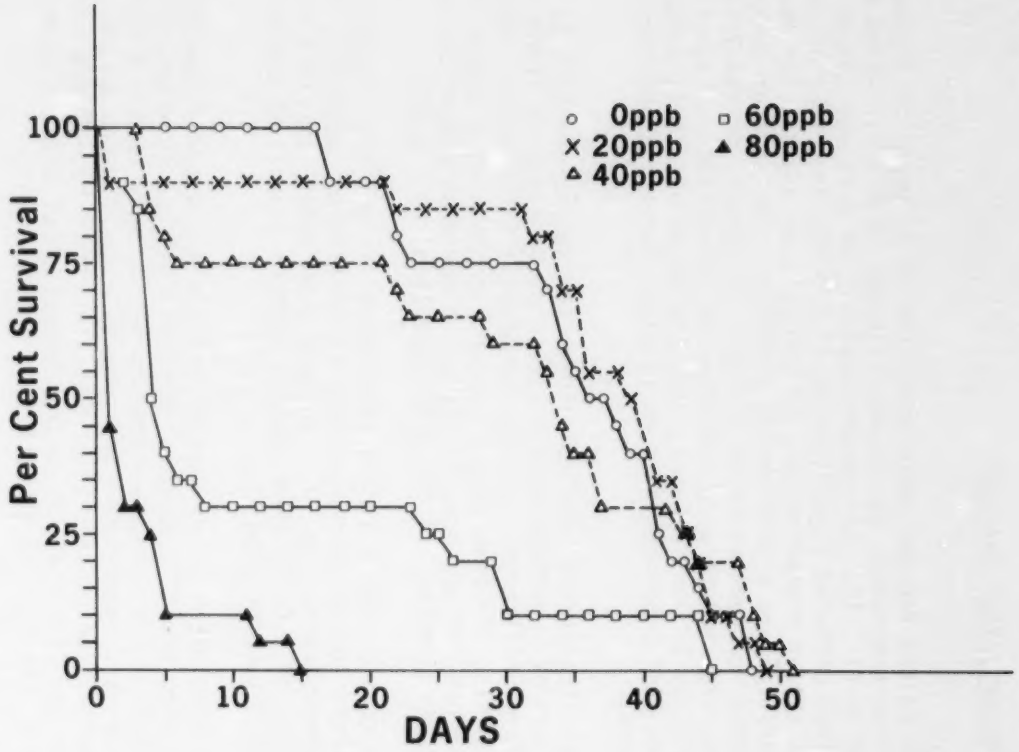

Figure 13. Combined survivorship curves (Experiments 1 and 2) for Daphnia pulex exposed to chronic copper stresses in the pond-water, algal system

would not have been detected in a 3-week chronic bioassay. Given the added stresses of a real world, it would seem possible that a copper concentration of 60 ppb would tip the scales sufficiently to eliminate the species. The problem of extrapolating from laboratory experiments to more complex, natural systems is, of course, the crucial problem. Sooner or later, if we want to protect species, as they exist in complex communities, we shall have to study stressed animals as components of more complex systems.

Reconstituted-Water, Algal Systems

Standard water--

Survival curves for two experiments run on D. magna in standard water made up from unaerated, Barnstead-filtered, double-distilled water are illustrated in Fig. 14. Chi-square analysis of survival curves indicate that survival differed significantly ($P = 0.05$) from that of control animals at copper concentrations of 5.0 ppb, or higher, for the 1/23 experiment. Survival was significantly different from that of control animals at a copper concentration of 7.5 ppb, or higher, in the 2/14 experiment. These experiments were run after we had noticed a reduction in longevity of animals in our filtered, standard water and it is probable that the animals were subjected to some chemical stress in addition to the copper stress.

Figure 15 illustrates survival curves for two experiments to determine the effect of a chronic copper stress on D. magna in standard water made up from unfiltered, aerated, double-distilled water. Survival was reduced significantly from that of control animals at a copper concentration of 15 ppb, or greater, in the 3/5 experiment and at concentrations of 20 ppb, or greater, in the 3/25 experiment ($P = 0.05$).

Survival curves, for a single experiment, in which D. magna was reared at different copper concentrations in unfiltered, unaerated standard water are presented in Fig. 16. In this experiment, survival was significantly less ($P = 0.05$) than that of control animals at copper concentrations of 5.0 ppb, or greater.

Although the longevity of control animals was somewhat variable, and the animals were probably exposed to other chemical stresses, the lowest concentration that reduced longevity varied only from 5.0 to 7.5 ppb in the 3 unaerated experiments and from 15.0 to 20.0 ppb in the 2 aerated experiments. Aeration of the distilled-water base appears not only to increase the life-span of control animals, but also to decrease the sensitivity of the animals to a chronic copper stress.

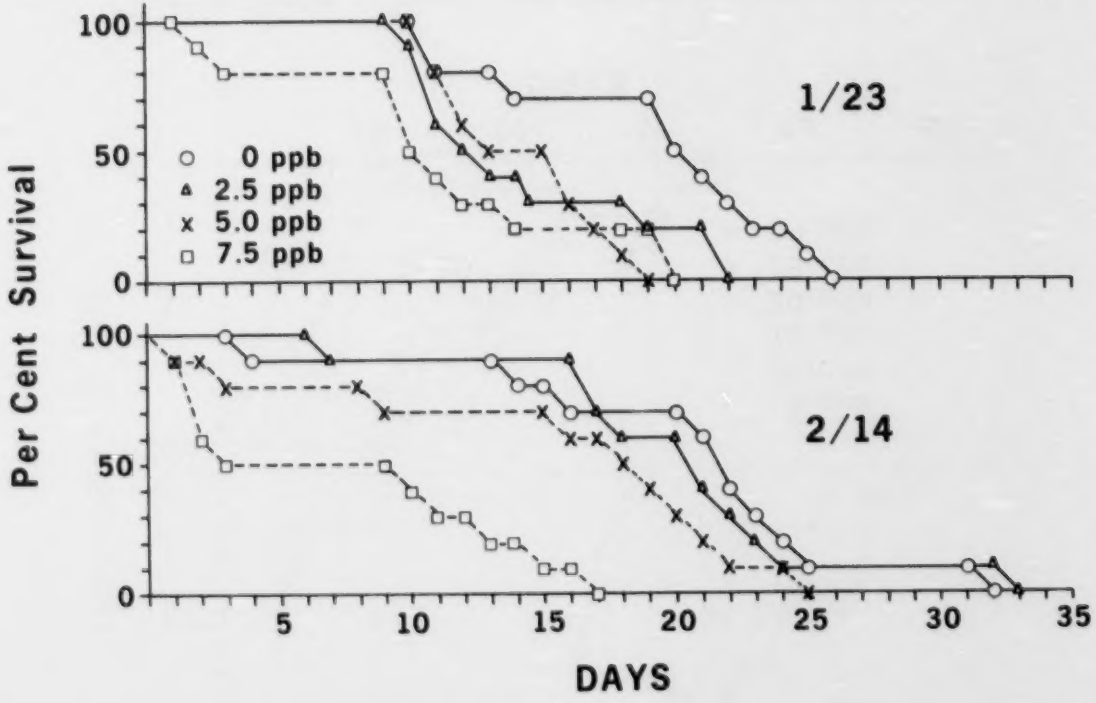

Figure 14. Survival of Daphnia magna fed algae and exposed to chronic copper stresses in filtered, unaerated standard water

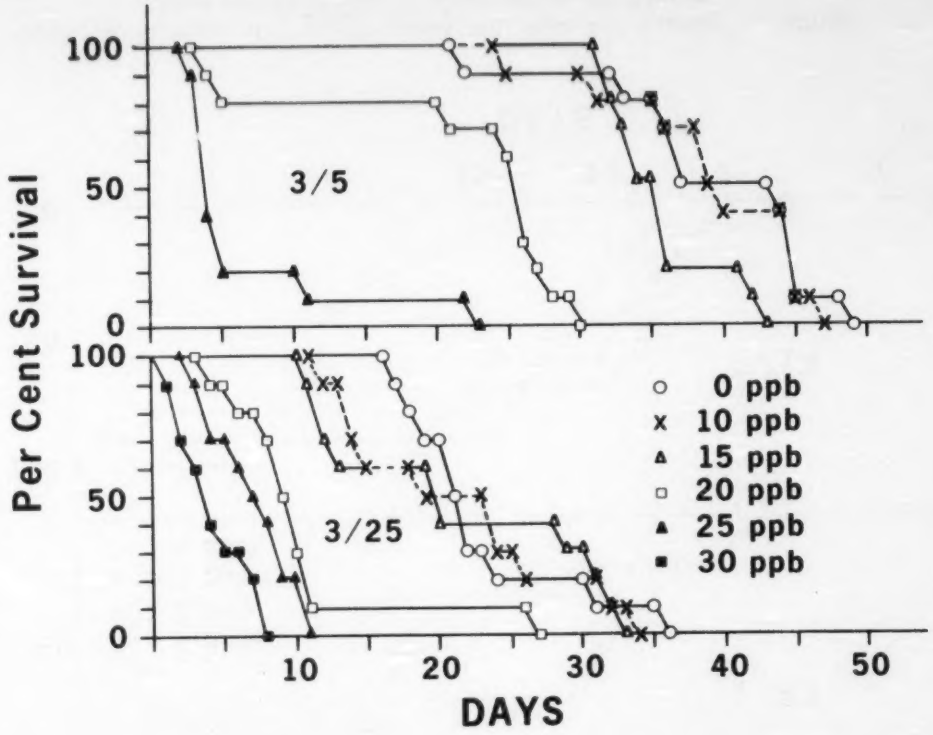

Figure 15. Survival of Daphnia magna fed algae and exposed to chronic copper stresses in unfiltered, aerated standard water

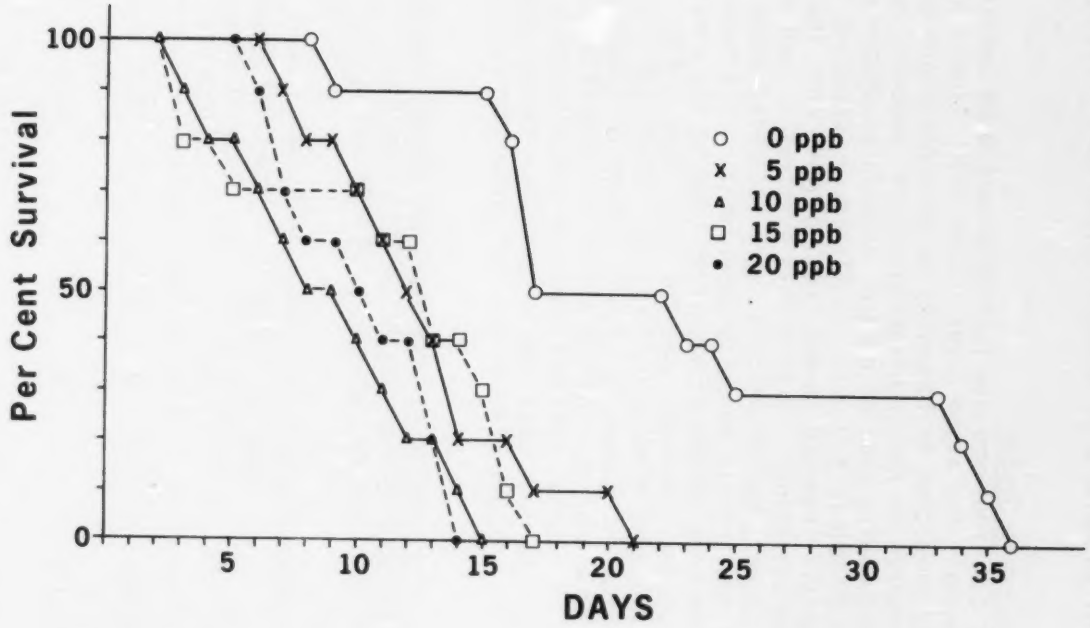

Figure 16. Survival of Daphnia magna fed algae and exposed to chronic copper stresses in unfiltered, unaerated standard water.

Medium water--

Only 2 chronic copper bioassays were run in medium water. These resulted in quite different results (Fig. 17). In the first experiment, all copper concentrations of 20 ppb, or more, caused a significant reduction in longevity ($P = 0.05$). In the second experiment, only copper concentrations of 80 and 100 ppb reduced longevity significantly ($P = 0.05$) below that of control animals. There was also a significant difference in longevity of control animals, with those of experiment 1 living much longer.

Pond-Water, Trout-Granule System--

Animals fed the trout-granule food were much more sensitive to copper than were animals fed algae (Figs. 6 and 18). Algal-fed animals did not exhibit any reduction in longevity at concentrations of 40 ppb, or less. Animals reared on the trout-granule food, however, had survival reduced significantly below that of control animals at copper concentrations greater than 20 ppb ($P = 0.05$). Initially, I suspected that the two foods differentially removed copper from solution and, thereby, altered effective concentration. To test this hypothesis, beakers containing 20 and 80 ppb copper, but no animals, were incubated under the same conditions used in the chronic toxicity experiments. The beakers were "fed," each day, the same ration of each food used in the chronic testing. The same batch of copper stock was used for both foods. After 72 hours, the contents were filtered through 0.45 micron filters (HA Millipore) and the filtrates analyzed for copper by atomic-absorption spectroscopy. The results (Table 9) show that the soluble copper concentrations were not different. It is, of course, possible that the concentrations of cupric ions did differ, but the fact that the 72-hour LC_{50} values were almost identical for the two systems (see section on acute toxicity of copper),

TABLE 9. RELATIONSHIP BETWEEN FOOD AND SOLUBLE COPPER CONCENTRATION REMAINING IN TEST BEAKERS AFTER 72 HRS OF INCUBATION IN POND WATER

Food	Nominal Copper Concentration (ppb)	Measured Copper Concentration (ppb)
Trout-Granule	20	15.8
Algae	20	15.4
Trout-Granule	80	46.5
Algae	80	40.5
Trout-Granule	80	49.2
Algae	80	48.0

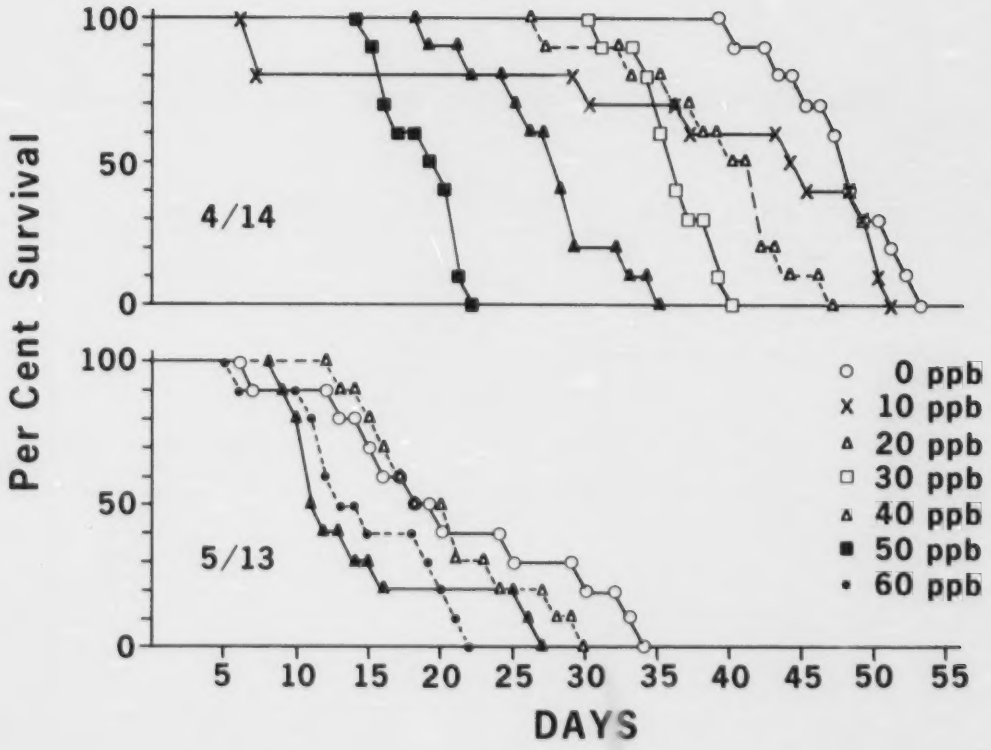

Figure 17. Survival of Daphnia magna fed algae and exposed to chronic copper stresses in medium water

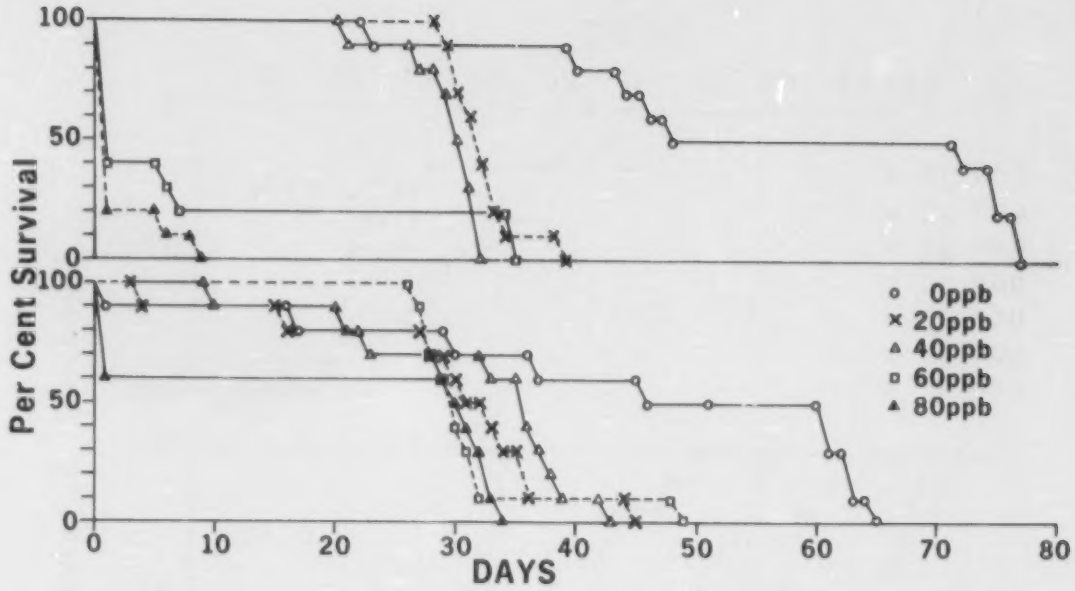

Figure 18. Effect of trout-granule food on the sensitivity of Daphnia magna to chronic copper stresses in pond water

strongly suggests that animals fed on the two foods were exposed to comparable concentrations of the toxic species of copper. If this is so, the greater sensitivity to copper of animals fed the trout-granule food must reflect a nutritional stress imposed in addition to the copper stress. The fact that control animals fed trout-granule food survived only about one-half as long as animals fed algae (Fig. 19) supports the conclusion that the trout-granule food is nutritionally inferior to the algal food. This nutritional inadequacy may not apply to all species of Daphnia. Dewey Bunting (personal communication) states that, although he cannot maintain continuous cultures of D. magna on trout food, he has no trouble in maintaining D. pulex on trout pellets.

Effect of a Chronic Copper Stress on Reproduction

Comparative Sensitivity of the Four Species--

The reproductive response of the four species to a chronic copper stress was somewhat more variable than was survival. Temporal changes in mean brood size, at each copper concentration, are illustrated in Figs. 20 through 23 for the four species. Although brood sizes oscillate, there is no evidence that any of the species approached a reproductive senility in old age. The oscillatory pattern does suggest that the results of an analysis of the impact of copper on brood size might vary, according to when the analysis was made during the life-span of the various cohorts. The question, thus, arises: how long should a chronic toxicity test be run, if effects of a stress on brood size are to be evaluated? To answer this question for a copper stress, sequential analyses of variance and Duncan's multiple-range tests were run on mean cumulative brood sizes from brood 1, through each consecutive brood, until the final brood had been incorporated into the analyses. In other words, the experiment "was stopped," after each brood had been produced, to determine what difference, if any, existed in mean brood size at the different copper concentrations. These sequential analyses for the four species are presented in Tables 10 through 13. It is obvious from Table 10 that D. magna is, reproductively, relatively insensitive to copper in the pond-water, algal system. At no point in time, from brood 1 through brood 33, was cumulative mean brood size at 80 ppb significantly different from that of control animals. Furthermore, copper was stimulatory up through a concentration of 60 ppb, i.e., mean brood sizes were always larger than the controls and, almost always, significantly so (Table 10). In D. magna, total reproductive output descreased at copper concentrations greater than 40 ppb (Table 9) only because the adults have a shorter life-span, and, therefore, produce fewer broods.

The young D. magna which developed in copper concentrations of 20, 40, 60 and 80 ppb were viable and cohorts of 10 were carried through 28-day F_1 and F_2 generations in their respective concentrations (Tables

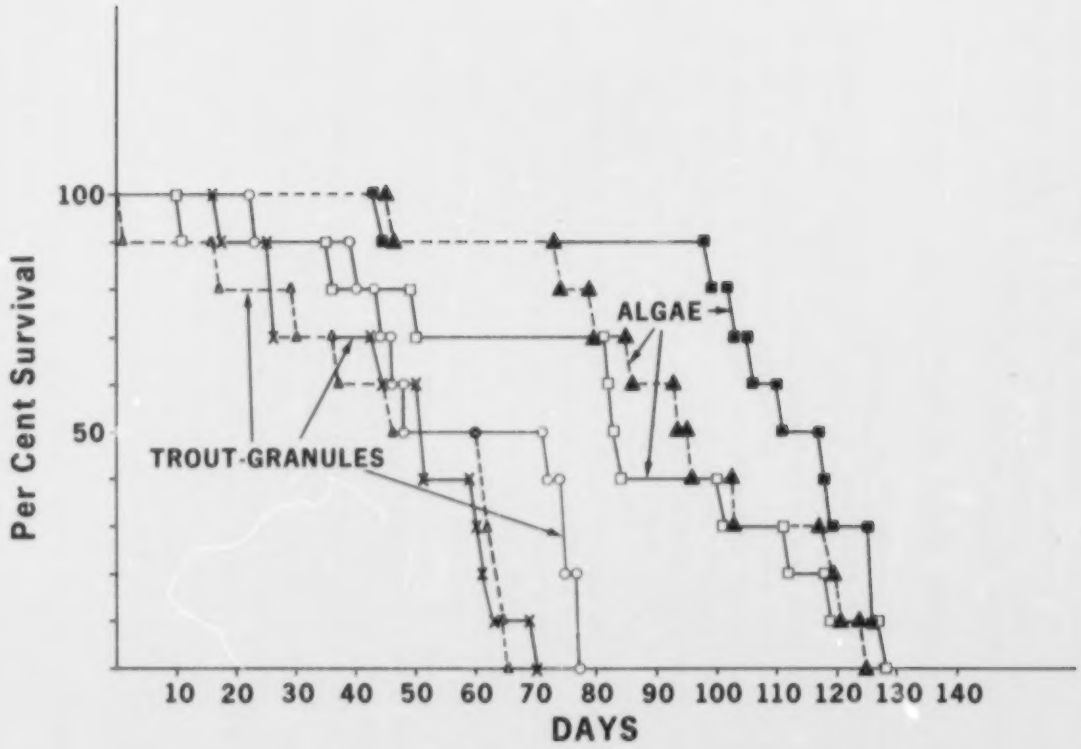

Figure 19. A comparison of longevity in Daphnia magna fed algae versus trout-granule food in pond water

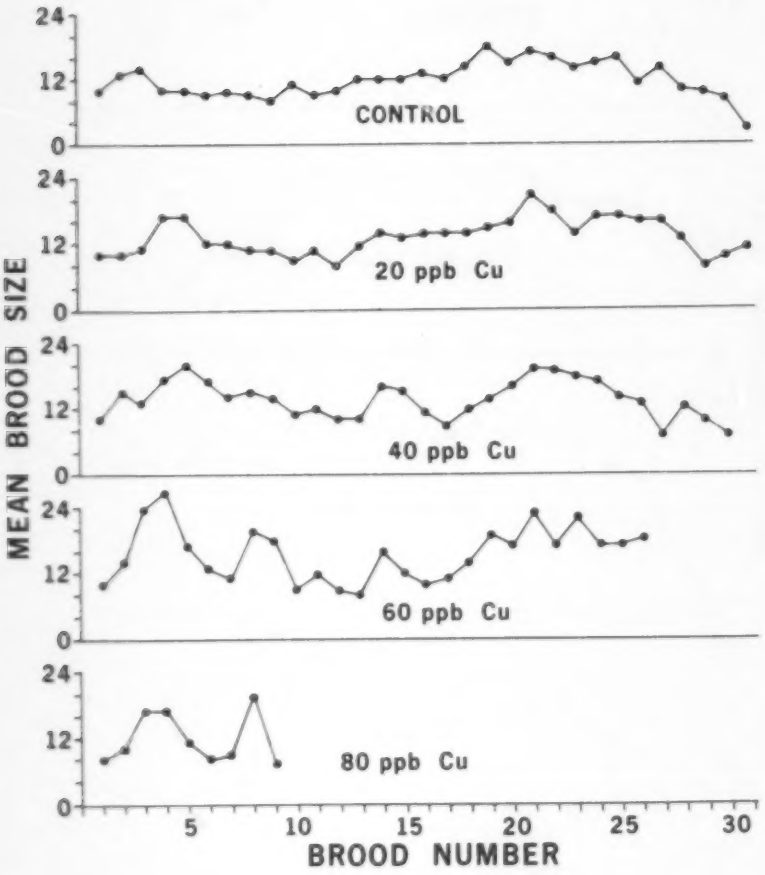

Figure 20. Temporal changes in the mean brood size of Daphnia magna exposed to chronic copper dosages

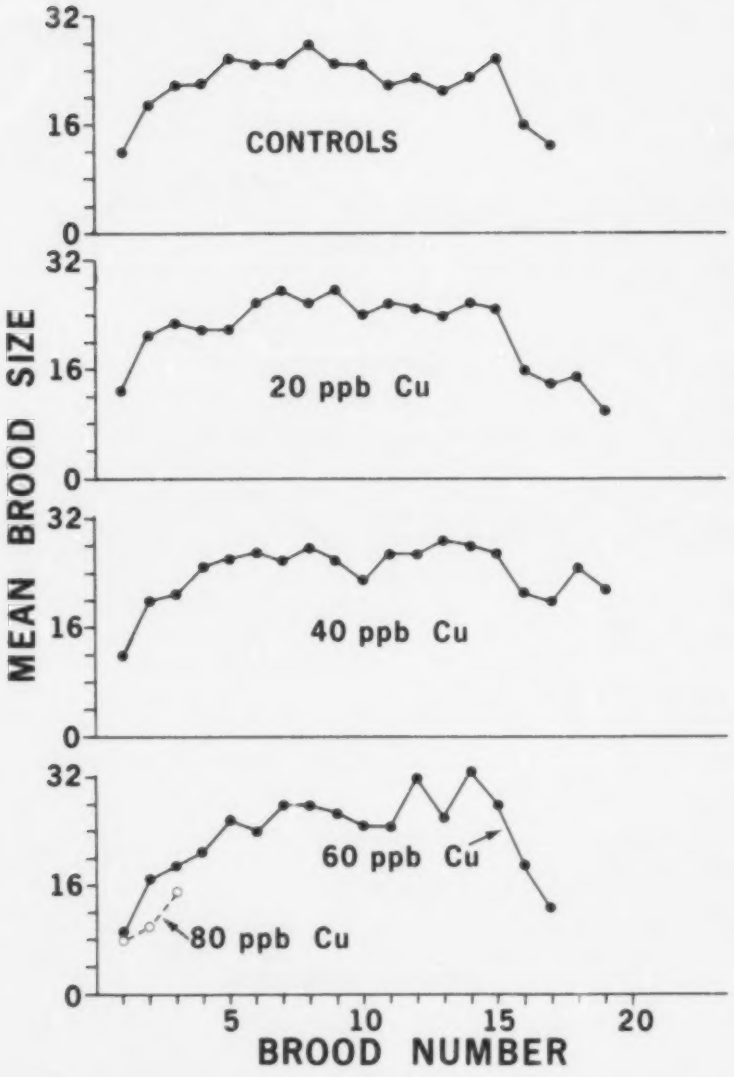

Figure 21. Temporal changes in the mean brood size of Daphnia pulex exposed to chronic copper dosages

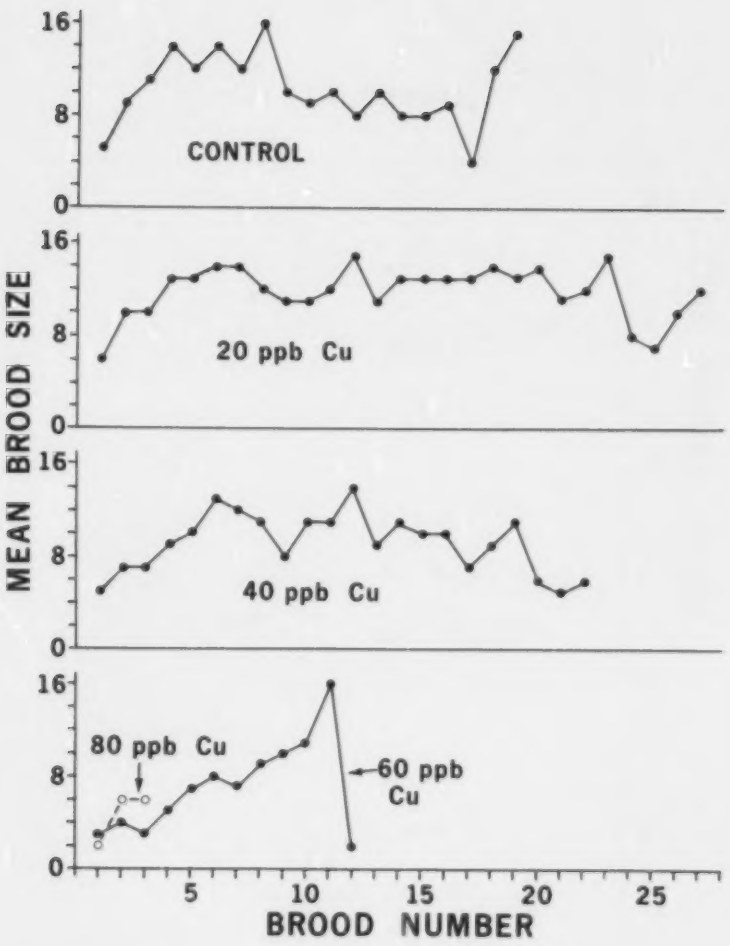

Figure 22. Temporal changes in mean brood sizes of Daphnia parvula exposed to chronic copper dosages

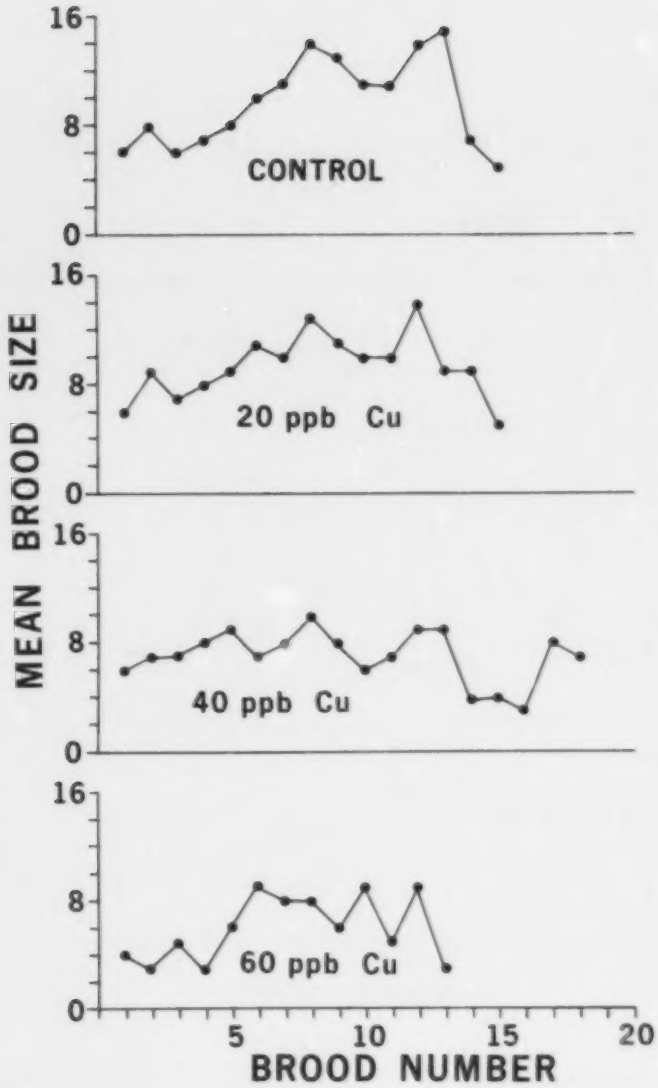

Figure 23. Temporal changes in mean brood sizes of Daphnia ambigua exposed to chronic copper dosages

TABLE 10. ANALYSES OF CUMULATIVE MEAN BROOD SIZES FOR *D. magna* EXPOSED TO FIVE COPPER CONCENTRATIONS IN THE POND-WATER, ALGAL SYSTEM[a]

Through Cumulative Broods	Nominal Copper Concentrations (ppb)				
1-4	40	60	0	20	80
5-7	60	40	20	80	0
8-11	60	40	20	80	0
12	60	40	80	20	0
13-14	60	40	80	20	0
15-24	60	40	20	80	0
25-27	60	40	20	0	80
28-30	60	40	20	80	0
31-33	60	40	20	80	0

[a]Brood sizes were significantly different ($P = 0.05$) in any two concentrations not mutually underlined. Brood sizes decrease in concentration, as sequenced, from left to right.

TABLE 11. ANALYSES OF CUMULATIVE MEAN BROOD SIZES FOR D. pulex EXPOSED TO FIVE COPPER CONCENTRATIONS IN THE POND-WATER, ALGAL SYSTEM[a]

Through Cumulative Broods	Nominal Copper Concentrations (ppb)				
1	20	0	40	60	80
2	20	0	40	60	80
3	20	40	0	60	80
4-10	20	40	0	60	80
11	20	40	60	0	80
12-15	40	20	60	0	80
16	40	20	60	0	80
17	40	60	20	0	80

[a]Brood sizes were significantly different (P = 0.05) in any two concentrations not mutually underlined. Brood sizes decrease in concentrations, as sequenced, from left to right.

TABLE 12. ANALYSES OF CUMULATIVE MEAN BROOD SIZE FOR D. parvula IN FIVE COPPER CONCENTRATIONS IN THE POND-WATER, ALGAL SYSTEM[a]

Through Cumulative Broods	Nominal Copper Concentrations (ppb)				
1	0	20	40	60	80
2	0	20	40	60	80
3-4	0	20	40	60	80
5	0	20	40	60	80
6-8	0	20	40	60	80
9-14	0	20	40	60	80
15-16	20	0	60	40	80
17	20	60	0	40	80
18-19	60	20	0	40	80
20-25	60	0	20	40	80
26-32	60	0	40	20	80

[a]Brood sizes were significantly different (P = 0.05) in any two concentrations not mutually underlined. Brood sizes decrease in concentrations, as sequenced, from left to right.

TABLE 13. ANALYSES OF CUMULATIVE MEAN BROOD SIZE FOR D. ambigua IN FOUR COPPER CONCENTRATIONS IN THE POND-WATER, ALGAL SYSTEM[a]

Through Cumulative Broods	Nominal Copper Concentrations (ppb)			
1	20	0	40	60
2-3	20	0	40	60
4	20	40	0	60
5	20	0	40	60
6	20	0	40	60
7-9	20	0	40	60
10-11	20	0	40	60
12	20	0	40	60
13-17	20	0	40	60

[a]Brood sizes were significantly different (P = 0.05) in any two concentrations not mutually underlined. Brood sizes decrease in concentrations, as sequenced, from left to right.

14 and 15. There is no evidence for either reduced viability or reduced fecundity in the F_1 and F_2 individuals when compared to the parental generation. This contradicts the conclusion of Hueck and Adema (1968) that copper is more toxic to second-generation daphnids than to their parents.

TABLE 14. TOTAL YOUNG AND MEAN BROOD SIZE FOR PARENTAL-, F_1- AND F_2-GENERATIONS OF D. magna ON A 28-DAY CHRONIC-COPPER STRESS IN THE POND-WATER, ALGAL SYSTEM

Copper (ppb)	Parental Total Young	Parental $\bar{x}$ Brood Size	F_1 Total Young	F_1 $\bar{x}$ Brood Size	F_2 Total Young	F_2 $\bar{x}$ Brood Size
0	951	11.1	839	14.2	375	15.0
20	1001	13.3	976	16.0	937	16.4
40	1218	16.9	1028	17.1	814	23.9
60	1300	17.8	674	18.7	1438	20.8
80	332	13.3	128	10.7	788	15.8

Based on 10, individually-reared, newborn animals started at each concentration and exmined daily for 28 days.

TABLE 15. PER CENT SURVIVAL OF PARENTAL, F_1 AND F_2 GENERATIONS OF D. magna AFTER 28 DAYS OF COPPER STRESS IN THE POND-WATER, ALGAL SYSTEM

Copper (ppb)	Parental	F_1	F_2
0	90	100	100
20	90	90	90
40	100	90	90
60	70	100	100
80	40	10	40

Each cohort consisted of 10, individually-reared-newborn animals

D. pulex consistently exhibited a significant reduction ($P = 0.05$) in mean brood size at copper concentrations of 80 ppb (Table 11). There were never significant differences in mean brood sizes at copper concen-

trations of 0, 20, 40 or 60 ppb. Copper stimulated fecundity at concentrations of 20, 40, and 60 ppb but these were usually not significantly larger than brood sizes of control animals (Table 11). Cumulative mean brood sizes through broods 16 and 17, however, were significantly larger at 40 ppb than for controls.

D. parvula exhibited a reduction in cumulative mean brood size only at copper concentrations of 80 ppb, or more, if the analysis was made subsequent to brood 8 (Table 12). If analyzed prior to brood 9, cumulative mean brood sizes in both 60 and 80 ppb were significantly smaller than those of control animals. Although cumulative mean brood sizes of animals reared at 20 and 60 ppb were frequently larger than those of control animals, the differences were never significant ($P = 0.05$).

Table 13 shows the analyses of the reproductive response of D. ambigua to copper stresses. Except for cumulative broods 1 and 6, the mean brood size was always significantly smaller ($P = 0.05$) at 60 ppb than at lower copper concentrations. Analyses of cumulative mean brood sizes from brood 10 through the last brood (17) indicate that mean brood sizes at 40 ppb are significantly smaller than those of control animals. Only the 20 ppb concentration caused a slight increase in brood size in D. ambigua, but this difference was never significant. Prior to brood 9, one would conclude that copper concentrations greater than 40 ppb caused a decrease in brood size of D. ambigua; after brood 9, one would conclude that copper concentrations in excess of 20 ppb resulted in a decrease in brood size.

In summary, D. magna was, reproductively, least sensitive to a chronic copper stress. Even at a copper concentration of 100 ppb, where only 4 broods were produced in three chronic experiments, the mean brood size, of those animals which had broods, was 10 (Table 9). Mean brood sizes at 40 and 60 ppb were usually significantly larger than those of 0 ppb. D. pulex was second least sensitive to copper. Only at a copper concentration of 80 ppb was there a significant reduction in brood size. D. parvula was third least sensitive; if analyzed after brood 8, there was a significant reduction in brood size at a copper concentration of 60 ppb. D. ambigua was most sensitive; mean brood sizes were, in all cases, significantly smaller at 60 ppb and at 40 ppb if analyzed after brood 10.

Sensitivity of D. magna in Reconstituted Waters

Standard Water--

The reproductive response of D. magna to a copper stress is more variable in filtered, unaerated standard water (Table 16) than is it in

TABLE 16. THE RELATIONSHIP BETWEEN MEAN BROOD SIZE AND COPPER CONCENTRATION FOR D. magna IN RECONSTITUTED WATERS

Experiment No.	Unaerated Standard Water		Aerated Standard Water		Unaerated Medium Water	
	Copper (ppb)	Mean Brood Size	Copper (ppb)	Mean Brood Size	Copper (ppb)	Mean Brood Size
1	0	11.7	0	11.0	0	12.9
	2.5	10.7[a]	5.0	11.0	10	16.1
	5.0	6.9	10.0	12.9	20	16.5
	7.5	3.0	15.0	15.9	30	16.7
	10.0	3.5	20.0	15.5	40	16.0
			25.0	16.8	50	13.1
2	0	9.9	0	10.2	0	18.1
	2.5	10.2	5.0	11.5	20	18.3
	5.0	10.0	10.0	12.4	40	14.4
	7.5	7.8	15.0	13.8	60	10.9
	10.0	9.9	20.0	11.1	80	5.4
			25.0	7.8		
3	0	14.6				
	5.0	8.5				
	10.0	7.3				
	15.0	9.0				
	20.0	4.5				

[a]Horizontal lines indicate that mean brood sizes above and below the line are significantly different (P = 0.05).

pond water (Table 8). In experiment 1, there was a significant reduction ($P = 0.05$) in mean brood size at copper concentrations above 2.5 ppb. There was a significant reduction in longevity at copper concentrations greater than 5.0 ppb (Fig. 14). In experiment 2, there were no significant differences in mean brood size at any of the copper concentrations, although survival was reduced at copper concentrations of 7.5 ppb, or greater (Fig. 14). In experiment 3, there was a significant reduction in mean brood size at copper concentrations greater than 15.0 ppb, but survival was significantly reduced at 5.0 ppb (Fig. 16). The stimulatory effect of copper on brood size that was so obvious in the pond-water experiments did not occur in unaerated standard water and reproduction was more sensitive than survival in 2 of the 3 experiments.

The relationship between copper, longevity and brood size in the unfiltered, aerated, standard-water bioassays is more similar to the results observed in the pond-water experiments (Table 16). In experiment 1, there was never a significant reduction in mean brood size at any copper concentration and mean brood sizes were significantly larger ($P = 0.05$) at 15, 20, and 25 ppb than at 0 ppb. Again, survival was more sensitive, being significantly reduced at concentrations greater than 10 ppb (Fig. 15).

In the second experiment, mean brood size was not significantly reduced ($P = 0.05$) at any copper concentration. Mean brood sizes were slightly larger at concentrations of 10 and 15 ppb than in 0 ppb, but never significantly so. Survival was significantly reduced at concentrations greater than 15 ppb (Fig. 15).

Medium Water--

Mean brood size for *D. magna* cultured in the medium-water, algal system are presented in Table 16. In the first experiment, copper had a slight stimulatory effect on mean brood size up to a concentration of 40 ppb but brood sizes were significantly larger ($P = 0.05$) than the controls only at 10 and 20 ppb. Mean brood size was not significantly smaller at 50 ppb copper than in the control. Since survival was significantly reduced at 20 ppb (Fig. 17), the results of this experiment agree with the pond-water experiments; i.e., survival is more sensitive to a copper stress than is reproduction.

Copper had no significant effect on mean brood size in the second experiment (Table 16). The sequential analyses of variance and multiple-range tests indicated that there were never significant differences in mean brood size at any point in the chronic experiment; survival, however, was significantly reduced at 80 ppb copper (Fig. 17).

Effect of Food-Type on Reproductive Sensitivity of D. magna in Pond Water

The trout-granule food not only reduced the life-span of D. magna, at all copper concentrations, it also increased the reproductive sensitivity of the species (Table 17). Tables 10 and 18 present the results of the sequential analyses of cumulative mean brood sizes in different copper concentrations for the algal- and the trout-granule foods. As discussed previously, copper did not cause a reduction in brood sizes in those algal-fed animals which lived long enough to reproduce. Table 18 clearly shows, however, that animals fed the trout-granule food exhibited significant reductions in mean brood size at copper concentrations of 20 ppb or more ($P = 0.05$). Animals, on this diet, are more sensitive to copper, using either survival or reproduction as an index. Also, the two indices agree that the maximum allowable concentration is less than 20 ppb for D. magna on the trout-granule diet. It should also be noted that, unlike the algal-fed animals, there was no stimulatory effect of copper on reproduction when the trout-granule food was used.

TABLE 17. EFFECT OF FOOD TYPE ON SURVIVAL AND REPRODUCTION OF *Daphnia magna* IN POND WATER[a]

Copper (ppb)	Mean Longevity (Days)		Mean Brood Size	
	Algal Food	Trout Food	Algal Food	Trout Food
0	86.2	50.5	11.9	21.4
20	99.7	31.3	13.6	14.8
40	88.5	30.0	14.3	11.7
60	46.7	31.2	15.1	9.6
80	16.6	13.6	12.4	8.2
100	5.1	1.5	13.0[b]	--

[a]Each mean is calculated from a cohort of 20 animals.

[b]Based on only two broods of 13 each.

Biesinger and Christensen (1972) evaluated the impact of a chronic copper stress on D. magna using the same trout-granule food. They state that there was a 16% reproductive impairment at a copper concentration of 20 ppb in Lake Superior water, which has a total alkalinity about one-fourth that of our pond water. However, they maintained their daphnids in groups of five and young were removed, and counted, only weekly. It was not possible, therefore, for them to differentiate between a direct impact of copper on reproduction and an indirect impact

TABLE 18. ANALYSES OF CUMULATIVE MEAN BROOD SIZE FOR *D. magna* IN FIVE COPPER CONCENTRATIONS WHEN FED TROUT-GRANULE FOOD IN POND WATER[a]

Through Cumulative Broods	Copper Concentrations (ppb)				
1	0	20	80	40	60
2	0	20	40	60	80
3	0	20	40	60	80
4-5	0	20	40	60	80
6-20	0	20	40	60	80

[a]Brood sizes were significantly different ($P = 0.05$) in any two concentrations not mutually underlined. Brood sizes decrease in concentrations, as sequenced, from left to right.

through a reduction in longevity and fewer broods. This made it impossible to determine what effect, if any, copper had on brood sizes. In the present study, for example, the total reproductive output from animals maintained at 60 ppb was considerably less than for control animals (Table 8) in spite of the fact that animals in the 60 ppb concentration had significantly larger broods than did control animals. The difference in reproductive output has nothing directly to do with reproduction, it simply reflects the shortened life span of animals maintained at 60 ppb. Biesinger and Christensen (1972) ran their chronic tests for only three weeks and they used a three-week LC_{50} as their estimate of the highest concentration that did not affect longevity. Use of both a three-week time limit and of LC_{50} values seem questionable as tools for determining the maximum concentration that does not significantly reduce survival. This, coupled with a lack of data showing the effect of copper on brood size, casts some doubt on their statement, ". . . reproductive impairment was found to be a more sensitive measure of toxicity than survival."

In the present study, animals reared on the trout-granule food also exhibited a retardation in reproductive development when cultivated in copper concentrations greater than 20 ppb. This was not true for animals fed the algal food (Table 19, Fig. 24). This is yet another indication that that trout-granule food makes the animals more sensitive to a copper stress.

TABLE 19. MEAN AGE AT REPRODUCTIVE MATURITY OF *D. magna* AS AFFECTED BY FOOD-TYPE AND COPPER CONCENTRATION IN POND WATER

Experiment	Food	Copper (ppb) 0	20	40	60
I	Algae	5.3	5.3	5.1	5.4
II	Algae	4.7	4.5	4.4	6.0
III	Algae	6.5	6.1	6.5	6.6
IV	Trout-Granule	5.6	7.5	8.6	13.4
V	Trout-Granule	6.2	6.6	7.7	11.1

EFFECT OF A CHRONIC COPPER STRESS ON THE INSTANTANEOUS RATE OF POPULATION GROWTH

The instantaneous rate of population growth, *r*, takes into account longevity and all facets of reproduction (e.g., age at reproductive

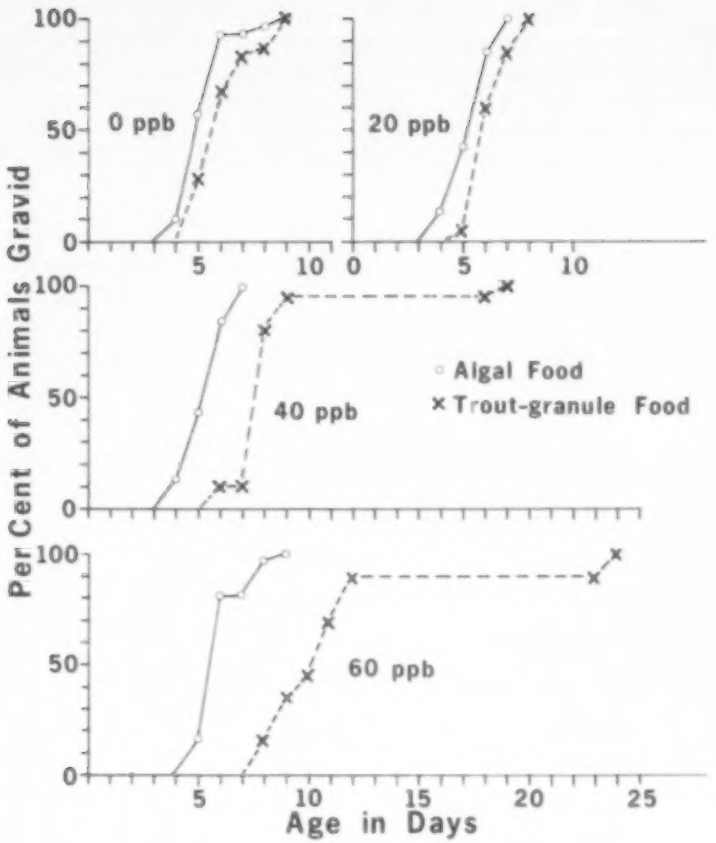

Figure 24. Effect of food type on age at reproductive maturity of Daphnia magna in pond water

maturity, age at reproductive senility, frequency of reproduction, and number of offspring per brood). Although other stresses may have different effects, in the present study, r-values, within a species, were largely determined by the interaction between longevity and brood size; copper did not affect age at reproductive maturity or frequency of reproduction in the pond-water, algal system. Differences between species were also related to species differences in frequency of reproduction and age at reproductive maturity.

Comparative Response of the Four Species

Species differences in the instantaneous rate of population growth are presented in Table 20. D. magna had the smallest r-values under control conditions. This is a reflection of: (1) longer maturation (D. magna produce their first young at an age of about 7 days, D. pulex at an age of 6 days, D. parvula and D. ambigua at an age of 5 days); (2) longer period of embryological development (3 days for D. magna and 2 days for the other species); and (3) D. pulex had larger broods than did D. magna. The early maturation and short period of embryological development of D. ambigua was somewhat negated by its producing the smallest broods of any of the four species.

TABLE 20. INSTANTANEOUS RATE OF POPULATION GROWTH (r) AS AFFECTED BY COPPER FOR FOUR SPECIES OF Daphnia IN THE POND-WATER, ALGAL SYSTEM

Copper (ppb)	D. magna	D. pulex	D. parvula	D. ambigua
0	0.373	0.498	0.423	0.459
20	0.405	0.469	0.424	0.454
40	0.409	0.453	0.390	0.432
60	0.325 [a]	0.230	*	0.233
80	0.287	*	*	*
100	*	*	*	*

[a]Horizontal lines indicate that the r-vales above and below the line are significantly different (P = 0.05).

*No reproduction occurred at these concentrations.

Table 20 shows that, except for D. magna, there is a significant reduction in r at concentrations above 40 ppb. This is due to both an increase in mortality and a reduction in brood size. In fact, reproduction is shut off completely for the three smaller species at a copper concentration of 80 ppb.

Effect of Food-Type on r

As was true for longevity and reproduction, r was affected at lower copper concentrations in the trout-granule system than in the algal system (Table 21). Algal-fed D. magna did not exhibit a reduction in r at copper concentrations less than 80 ppb, whereas D. magna fed on the trout-granule diet exhibited a reduction in r at 60 ppb (Tables 20 and 21). Under all conditions, in the present study, r was a less sensitive index of copper stress than longevity. This may, however, not be true for other stresses. It seems possible that conditions could exist where r was more sensitive than either longevity or brood size. Such a situation might involve stresses that affect age at reproductive maturity or frequency of reproduction.

TABLE 21. EFFECT OF A COPPER STRESS ON THE INSTANTANEOUS RATE OF POPULATION GROWTH OF D. magna IN THE TROUT-GRANULE, POND-WATER SYSTEM

Copper (ppb)	Experiment 1	Experiment 2	Mean
0	0.384	0.331	0.358
10		0.339	
20	0.318	0.291	0.305
40	0.237	0.259	0.248
60	0.211 [a]	0.211	0.211
70		0.191	
80	0.070	0.162	0.116
90		0.067	

[a]Horizontal lines indicate that r-values above and below the line are significantly different (P = 0.05).

A Comparison of Longevity, Brood Size, and r as Indices of Copper Stress

Table 22 compares the maximum allowable toxicant concentration for copper that would be permitted in the pond-water, algal system using

TABLE 22. MAXIMUM ALLOWABLE TOXICANT CONCENTRATION OF COPPER (PPB) FOR FOUR SPECIES OF *Daphnia* BASED ON LONGEVITY, BROOD SIZE AND *r*

Species	MATC Based on		
	Longevity	Brood Size	*r*
D. magna	40	80+	60
D. pulex	40	60	40
D. parvula	40	60	40
D. ambigua	40	40	40

longevity, brood size, and *r* as indices. Longevity and *r* both predict that the three smaller species would be adversely affected at copper concentrations greater than 40 ppb. For *D. magna*, however, longevity is a more sensitive index than is *r*. This reflects the stimulatory effect of copper on brood size in *D. magna*, which more than offsets the reduced longevities at 60 ppb. Brood-size response to a chronic copper stress is more variable than either *r* or longevity. In *D. magna*, *D. pulex*, and *D. parvula*, brood size is less sensitive to a copper stress than either *r* or longevity. For *D. magna*, use of brood size as an index would predict an MATC of copper twice that predicted by longevity. For *D. ambigua*, the three indices are in agreement in predicting the MATC to be 40 ppb copper.

An Evaluation of the Application-Factor Concept

Comparison of Interspecific Application Factors--

Application factors, using only those 72-hr LC_{50} values from parallel concentration-response curves (Table 4) and MATC values calculated from chi-square analyses of combined survival curves for two chronic tests for each species, are presented in Table 23. Survival was used as the best estimate of MATC because it was as, or more, sensitive than brood size or *r* for each of the four species. These application factors vary only from 0.46 for *D. magna* to 0.59 for *D. ambigua*.

Application factors which have been published for fish (e.g., Mount, 1968; Mount and Stephan, 1969; and McKim and Benoit, 1971) have been calculated using the mean of several, independently-determined LC_{50} values. The statistical validity of this is questionable (Finney, 1971), since in none of the publications is there any evidence that the LC_{50} values are from parallel concentration-response curves.

TABLE 23. APPLICATION FACTORS OF COPPER FOR FOUR SPECIES OF Daphnia IN THE POND-WATER, ALGAL SYSTEM

Species	MATC (Longevity)	72-Hr LC_{50}	Application Factor
D. magna	40	85.1	0.46
D. pulex	40	86.0	0.47
D. parvula	40	72.0	0.56
D. ambigua	40	67.7	0.59

The use of mean LC_{50} values for calculating application factors in the present study does not, however, significantly change the results. Using mean, 72-hr LC_{50} values for each species, the factors still vary only from 0.46 for D. magna to 0.62 for D. parvula. A one-way, complete random design ANOVA indicates that these values are not significantly different ($P = 0.05$).

I can find no references in the literature to application factors for Daphnia. Copper application factors, as reported in the literature for fish, are much smaller than those we have observed for Daphnia. For example, in a study of brook trout, the application factor for copper was estimated to lie between 0.10 and 0.17 (McKim and Benoit, 1971). Mount (1968) and Mount and Stephan (1969), working with fathead minnows, estimated the application factor for copper to be between 0.03 and 0.07 in hard water and between 0.14 and 0.24 in soft water. The larger application factors for the daphnids may be due to the fact that an acute test comprises a much greater fraction of the total life cycle of daphnids than of the life cycle of fishes.

Effects of Water Chemistry on the Application Factor--

The acute and chronic toxicity of copper was more variable in reconstituted waters than in pond water (Table 24) and, therefore, application factors were more variable for D. magna. This greater variability was probably caused by several factors. In standard water, for example, the very low test concentrations, differing by only 2.5 to 5.0 ppb, are probably, in themselves, contributing to the variability. In the two filtered-water experiments a shift of the MATC, from only 2.5 to 5.0 ppb, doubled the application-factor estimate. Also, as previously pointed out, there was a secondary, and variable, chemical stress in some, or all, of the reconstituted-water experiments. A reproducibly-adequate, distilled-water base should reduce the test variability. There is a

suggestion of this in the two experiments with aerated standard water (Table 24). Not only are the application factors more similar for these two experiments, they are also much closer to the application factors calculated for D. magna in pond water.

TABLE 24. EFFECT OF WATER- AND FOOD-TYPE ON COPPER APPLICATION FACTORS FOR Daphnia magna

Water	Food	72-hr LC_{50}	MATC	Application Factor
Pond	Algae	88.8	40	0.45
Pond	Algae	85.0	40	0.47
Pond	Algae	81.5	40	0.49
Pond	Trout-Pellet	81.4	10	0.12
Pond	Trout-Pellet	85.3	10	0.12
Filt. Std.	Algae	13.0	2.5	0.19
Filt. Std.	Algae	13.0	5.0	0.38
Unfilt. Std.	Algae	25.5	2.5	0.10
Aer. Std.	Algae	31.6	10	0.32
Aer. Std.	Algae	31.6	15	0.47
Medium	Algae	101.4	10	0.10
Medium	Algae	88.1	60	0.68

The greatest disparity in results occurred in the two experiments with reconstituted medium water. Estimates of both 72-hr LC_{50} and MATC values were very different for the two experiments. Again, I feel that this is due to variability in the quality of our distilled water.

Effect of Food-Type on the Application Factor--

The trout-granule food greatly increased the sensitivity of D. magna to a chronic copper stress, but did not significantly alter the LC_{50} values from those obtained with the algal food (Table 24). As a consequence, the difference between LC_{50} and MATC was increased and the application factor was much smaller for animals on the trout-granule food. The two tests with the trout-granule food, like the three tests with algal food, were very consistent. The smaller application factor, like those observed in some reconstituted-water experiments, may reflect

an added stress, in this case, a nutritional stress. It is also interesting to speculate, since the chronic response exhibited a striking change and the acute response did not, that the mode of action of copper may be different in acute and chronic situations.

SECTION VI

REFERENCES

Analytical Qual. Control Lab. 1971. Methods for Chemical Analysis of Water and Wastes. U. S. E. P. A., Cinn., OH. 312 pp.

Biesinger, K. A. and G. M. Christensen. 1972. Effects of various metals on survival, growth, reproduction, and metabolism of Daphnia magna. J. Fish. Res. Bd. Canada. 29:1691-1700.

Brooks, J. L. 1957. The Systematics of North American Daphnia. Mem. Conn. Acad. Arts Sci. XIII. Yale U. Press, New Haven, Conn. 180 pp.

Christian, G. D. and F. J. Feldman. 1970. Atomic Absorption Spectroscopy. Applications in Agriculture, Biology, and Medicine. Wiley-Interscience, New York, N. Y. 490 pp.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics. 11:1-42.

Finney, D. J. 1971. Probit Analysis. Cambridge U. Press, London. 333 pp.

Hueck, H. J. and D. M. Adema. 1968. Toxicological investigations in an artificial ecosystem. A progress report on copper toxicity towards algae and daphniae. Helgoländer wiss. Meersunters. 17: 188-199.

Kemp, H. T., J. P. Abrams, and R. C. Overbeck. 1971. Effects of chemicals on aquatic life: Water quality criteria data book. Vol. 3. U. S. E. P. A. Water Poll. Contr. Res. Serv. 18050 GWV05/71. U. S. Govt. Print. Off., Washington, D. C. 528 pp.

Mantel, N. 1966. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother. Repts. 50:163-170.

Marking, Leif L. 1969. Toxicity of quinaldine to selected fishes. Invest. Fish Control No. 23. Bur. Sport Fish. Wildl., Washington, D. C. 10 pp.

McKim, J. M. and D. A. Benoit. 1971. Effects of long-term exposures to copper on survival, growth, and reproduction of brook trout (Salvelinus fontinalis). J. Fish. Res. Bd. Canada. 28:655-662.

Mount, D. I. 1968. Chronic toxicity of copper to fathead minnow (Pimephalas promelas, Rafinesque). Water Res. 2:215-223.

Mount, D. I. and C. E. Stephan. 1969. Chronic toxicity of copper to the fathead minnow (Pimephalas promelas) in soft water. J. Fish. Res. Bd. Canada. 26:2449-2457.

Murphy, J. S. 1970. A general method for the monoxenic cultivation of the Daphnidae. Biol. Bull. 139:321-332.

Pagenkopf, G. K., R. C. Russo, and R. V. Thurston. 1974. Effect of complexation on toxicity of copper to fishes. J. Fish. Res. Bd. Canada. 31:462-465.

Stephan, C. E. (Ed.). 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. Ecol. Res. Ser. No. EPA-660/3-75-009. U. S. Environmental Protection Agency, Duluth, Minn. 61 pp.

Stiff, M. J. 1971. Copper/bicarbonate equilibria in solutions of bicarbonate ion at concentrations similar to those found in natural waters. Water Res. 5:171-176.